Drugging Transcription: Exploring Roles of CDK8 From Cancer to Immunity

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Drugging Transcription: Exploring Roles of CDK8 from Cancer to Immunity

A Dissertation Presented by

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In Partial Fulfillment for the Requirements for a Degree of

Doctorate of Philosophy in Chemical Biology

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Abstract:

Kinases are cellular enzymes, integral in control of cellular growth and development. Kinases are frequently mutated or dysregulated in various disease states, such as cancer, neurological diseases, and immune disorders. The cyclin dependent kinase (CDK) family is a large family of kinases named for their members’ close association with obligate regulatory binding partners known as cyclins. CDKs can be grouped primarily into CDKs involved in regulation of the cell cycle, or regulation of transcription.

CDK8 is one of the transcriptional CDKs, part of the eukaryotic transcriptional coactivator, Mediator. CDK8 has distinct structural roles as part of Mediator, as deletion of CDK8 or its submodule leads to impairment of transcription and is embryonically lethal. CDK8 has been implicated in playing oncogenic roles in numerous cancers, including Wnt/β-catenin dependent colorectal cancer and gastric adenocarcinoma. However, this link emerged through genetic studies via silencing of CDK8 with siRNA, supporting the importance of structural presence of CDK8, but not confirming the importance of CDK8 kinase activity.

My initial studies suggest that while CDK8 knock down may be anti-proliferative, CDK8 kinase inhibition is not. CDK8 inhibition had no anti-proliferative impact on the subset colorectal cells previously reported as sensitive to CDK8 siRNA. However, a clear role for CDK8 kinase activity in regulation of select transcriptional networks is present in the literature, wherein CDK8 regulates gene expression through direct phosphorylation of transcription factors, including STAT1, SMADs, E2F1, HIF1α, p53, serum response network, among others. Likewise, I observed CDK8 inhibition affected a subset of genes controlled by IFNγ activation, wherein CDK8 phosphorylates STAT1 on a regulatory serine, S727, in its transactivation domain. This supports a divergence in the role CDK8 plays structurally versus catalytically. While steric presence of CDK8 with Mediator is necessary for maintaining basal transcription, the kinase activity is important when the cell needs to respond to stimuli.
In this vein, I explored areas where responses to external cellular stimuli were critical, alighting on immune activation. We utilized a small molecule screen in mouse primary bone marrow derived dendritic cells (BMDCs), identifying a previously unappreciated role for CDK8 inhibition in innate immune activation. Therein, inhibition of CDK8 leads to an enhancement of production of the anti-inflammatory cytokine IL-10, while reducing production of pro-inflammatory cytokines such as TNFα and IL-6 in activated myeloid cells. This lead to the conclusion CDK8 inhibition promotes tolerance in the innate immune compartment. Interestingly, the mechanistic underpinnings of this result occur through CDK8 driven phosphorylation of the transcription factor c-Jun, on a regulatory Serine, Ser243. While this finding suggested a critical role for CDK8 in innate immunity, it does also bring to question the therapeutic value of CDK8 inhibition as an oncogenic target, where increased tolerance and IL-10 production may be detrimental.

From there I endeavored to determine if this tolerogenic effect was restricted to the innate immune compartment, or if CDK8 played any role in the adaptive immunity. To explore this I looked at the impact of CDK8 inhibition on polarization of CD4+ T helper cells towards pro-inflammatory (Th1/Th17) or anti-inflammatory (Treg) lineages. Interestingly, CDK8 inhibition selectively promoted differentiation of naïve CD62L+/CD4+ T helper cells to anti-inflammatory FOXP3+/CD4+ Tregs, which were functional in both in vitro and in vivo models. While more work is left to fully elucidate CDK8’s mechanistic role in CD4+ T helper cell differentiation, this work further supports a general role for CDK8 in control of inflammation and tolerance in both the innate and adaptive immune compartments.
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As most of you probably know, getting a Ph.D. is... an *involved* process—more so than I knew when starting. As you read this thesis—serving as an accounting of how I have spent my time navigating through the minefields of cancer biology and immunology—I’ll be the first to admit that I never would have made it here but for the support and guidance that I have had through the years. My hands held the pipettes, but I have had so many amazing people, minds, and personalities that have encouraged me through the ups, downs and twist-about. So, while the next few (dozens…) of pages will be about the work that I have completed to warrant getting this degree, I wanted to ensure that there was a call out to the support system that has led me here.

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Chapter 1: Introduction to Kinases and Kinase Inhibition
1.1. **History of Kinase Inhibitors, probes and drugs:**
Kinases are enzymes which bind and utilize adenosine triphosphate (ATP) to catalyze the transfer of a phosphate group from ATP to a substrate (protein or small molecule), functioning as key regulators of cellular signaling and signal transduction pathways. The addition of a highly negatively charged phosphate group to protein targets induces a significant change in protein charge and conformation, altering protein function. Kinases are integral in control of cellular growth and development, thus are frequently mutated or dysregulated in various disease states, such as cancer, neurological disease, and immune disorders.

Kinases exhibit a conserved two lobed structure, with an N-terminal and a C-terminal lobe connected by a hinge domain. Additionally, there are residues which binds Mg$^{2+}$ and coordinates the highly negatively charged phosphate groups of ATP. The kinase binding pocket also contain amino acid residues which allow for favorable binding of the adenosine moiety of ATP. Since kinases innately bind a small molecule in the form of ATP, there is potential for developing small molecule inhibitors which compete for binding in active site and inhibit enzymatic function. Utilizing this strategy has allowed for the development of various types of kinase inhibitors. Currently, there are numerous approved kinase inhibitors as well as many that are in various phases of clinical trials.

Kinase inhibitors historically fall into three main categories, Type I substrate competitive inhibitors which directly compete for active site occupancy, Type II inhibitors which bind the active site and lock the kinase in the inactive (DFG in) conformation, and allosteric inhibitors which bind in a secondary pocket and indirectly regulate active site conformation. However, there are emerging groups of kinase inhibitors which
function through alternative mechanisms, such as covalent inhibitors and E3 ligase recruiters and degraders. These may share structural features with the other Type I, II or allosteric inhibitors, but function differently in cells or whole organisms.20, 27

1.1.1. Type I Inhibitors
Type I kinase inhibitors function as ATP mimetics, competing for ATP binding site occupancy to the active kinase conformation (DFG-out).21 Type I inhibitors generally have heterocyclic systems that functionally mimic the adenosine group of ATP. One of the first discovered kinase inhibitors, staurosporine 28, 29, a natural product, typifies this multi-ring aromatic system. However, staruosporine acts as a pan-kinase inhibitor with poor selectivity across the kinome, in fact binding most ATP dependent enzymes.30 Thus staurosporine was found to be highly toxic, limiting is therapeutic value. However, it provided a model for development of Type I ATP competitive inhibitors.

Examining the binding of an inhibitor in the kinase active site allows for identification structure activity relationships (SAR), determining which parts of the molecule are essential for binding, and which may be modified to allow for additional activity.1 Moreover, the development of kinase panels for inhibitor profiling has allowed for determination of the selectivity of desired inhibitors across the kinome.31 This has allowed for generation of selective Type I kinase inhibitors which have more favorable therapeutic indexes.28, 29, 32-34

1.1.2. Type II Inhibitors
While initially, most kinase inhibitors were thought to bind similarly to ATP, in the active form of the kinase, crystallization of these inhibitors with their target kinase led to the discovery that some inhibitors, such as sorafenib, bound to the inactive kinase
These Type II kinase inhibitors instead locked the kinase in the inactive confirmation, where the DFG motif is pointed inward. These Type II kinase inhibitors instead locked the kinase in the inactive confirmation, where the DFG motif is pointed inward.  

1.1.3. Allosteric Inhibitors

Additionally, there are multiple examples of kinase inhibitors that do not bind in the ATP pocket. Allosteric kinase inhibitors function by binding in non-ATP sites on the kinase and indirectly regulating ATP or downstream substrate binding thus to inhibit kinase function. Since the allosteric inhibitors are not ATP competitive, they can often avoid common active site mutations that lead to inhibitor resistance. Allosteric inhibitors can be challenging to discover, as it is uncommon to have an obvious allosteric pocket that can regulate the kinase function. However, since these allosteric pockets tend to be more unique to a one kinase from the highly conserved active sites, they lead to increased selectivity and minimal toxicity. Successes have come from MEK1/MEK2 inhibitors and inhibitors to the myristoyl binding pocket of some kinases.

1.1.4. Emerging Inhibitor types

Due to the highly-conserved nature of the kinases active site, selectivity can be a challenge. Less selective inhibitors often can have significant off target effects that can lead to undesirable toxicity, limiting their therapeutic potential. An emerging method to combat this toxicity exploits unique differences in the location of moderately active sulphur in cysteine residues in and located near the kinase active site. Using docking models and crystal structures it is possible to determine if the location of a cysteine residue is in a position that allows it to be covalently modified with a weak electrophile that is brought near upon inhibitor binding. In such a manner, it is possible to achieve additional selectivity through covalent binding at a lower concentration, as binding will be
selectivity enriched in kinases which can be covalently modified. While there has been worry about how well these covalent modifiers will be tolerated, certain kinases have shown promise in targeting this way. CDK7, EGFR, HER3, Jak3.1, 20, 39, 40

Additionally, recruitment of cellular machinery which naturally control the degradation of protein targets has allowed for alternative methods to regulate kinase activity.41 Since kinase function can involve both catalytic and structural activities, often times the inhibition of a kinase does not faithfully mimic genetic knock down. By linking a kinase inhibitor to the small molecule thalidomide via a flexible linker it is possible to recruit the thalidomide binding protein, cereblon and the E3-ligase machinery. Once recruited the E3 ligase can tag the kinase for degradation, thus not just inhibiting the catalytic activity, but chemically knocking down the protein.41
Chapter 2: Cyclin Dependent Kinases
2.1. Introduction to CDK family

The cyclin dependent kinase (CDK) family is a large family of kinases named for their members’ close association with obligate regulatory binding partners known as cyclins.\(^2\) In the absence of cyclin-binding, CDKs are catalytically inactive.\(^42\) However, binding to their cyclin partner stabilizes the CDK catalytic site and positions the conserved T loop so as not to occlude the active site. Additionally, the cyclin partner stabilizes substrate coordination into the active site (Figure 2.1).\(^43,44\) CDKs regulate a diverse array of cellular processes, however they can primarily be grouped into CDKs which are involved in regulation of the cell cycle, or regulation of transcription. Additional atypical CDKs have highly varied roles, but are less well studied.

Cyclins comprise a diverse class of proteins with little sequence conservation beyond the structurally conserved four helix cyclin box which mediates the CDK/cyclin interaction. The non-conserved region of each cyclin aids both in the selectivity of CDK/Cyclin binding and the downstream substrate interactions.\(^2\) This is especially important as certain CDKs can bind multiple cyclins, which differentially modulate their biological function, as evidenced during important cell cycle transitions (Figure 2.2).\(^2\)

In the cell cycle cyclins, while CDK expression is usually constant throughout the cell cycle, the cyclin levels are more tightly regulated and vastly fluctuate. Change in binding partner cyclin levels is the primary method through which cell-cycle CDK activity is regulated. However, phosphorylation of the activation domain on the T-loop tightens the CDK/Cyclin interaction and allows for an additional level of regulation.\(^43\) Depending on the CDK, T-loop phosphorylation can be essential for CDK activity, or might not be present at all.
2.1.1. Cell cycle regulation CDKs 2-6

CDKs were initially discovered as key regulators of progression through the phases of the cell cycle. In this manner, CDK activity was controlled via alternation of binding to cyclins, whose concentration oscillated with progression through the phases of the cell cycle. Alternation of Cyclin binding determines CDK substrate specificity (Figure 2.2). These cell cycle CDKs consist of three CDKs which regulate progression through various interphase steps (CDK 2, CDK4, and CDK6) as well as mitotic CDK (CDK1) which tightly controls progression through mitosis, as well as the less well studied CDK3 which appears important in regulating entrance and exit from G₀. Mis-regulation of the cell cycle CDKs can lead to aberrant division, genomic instability and chromosomal...
instability as these CDKs function at checkpoints to prevent division with DNA damage or improper chromosomal segregation.\textsuperscript{2, 44}

The function of the cell cycle CDKs is regulated by binding cyclins of four classes (type A, B, D and E cyclins).\textsuperscript{42} Cyclin concentration changes throughout the phases of the cell cycle, functioning as a cellular oscillating clock, and controlling the CDK substrate specificity. Degradation and alternation of the bound cyclins control which downstream CDK targets are phosphorylated, and allow for progression through the cell cycle.\textsuperscript{45} (Figure 2.2)

Interestingly, CDK1 is the only cell cycle CDK essential for development, being most closest related to the yeast cell cycle CDK, Cdc28.\textsuperscript{44} Because of the promiscuity with cyclin binding, CDK1 can substitute for all interphase CDKs in all but a select few cell subtypes. Animal cell cycle CDKs became further specialized, acquiring the additional interphase CDKs (2, 3, 4 and 6) which allowed for more fine-tuned control of division in multiple cell types.\textsuperscript{44} Loss of interphase CDKs is not embryonically lethal and organismal development is possible. However, it does prevent complete development in cell specific compartments. Loss of CDK2 leads to improper development of cardiomyocytes, and loss of CDK4/6 has a negative impact to the development of hematopoietic cells.\textsuperscript{2, 44}
Figure 2.2: Transition between the cell cycle phases is controlled by the oscillation in concentration of Cyclins. Cyclin binding, controls activity of their CDK binding partner, regulating their downstream substrate specificity. This allows for phosphorylation of proteins controlling transition between cell cycle phases. In yeast, there is only one cell cycle CDK, the mitotic Cdc28, most closely related to CDK1. Mammals have evolved additional interphase specific CDKs which allow for more fine-tuned control of the cell cycle and additional control in specific cell types. However, the mitotic CDK1 is still the only cell cycle CDK necessary for embryonic development.

2.1.2. Transcriptional CDKs

The second major function of CDKs is in regulation of transcription. Transcriptional CDKs vary from their cell cycle related brethren as they preferential bind to one cyclin. Additionally, the cyclin partners of transcriptional CDKs do not tend to fluctuate, thus they remain bound to their CDK. Moreover, while the cell cycle CDKs exhibit some level of specialization, the only essential cell cycle member is CDK1.44-46 Contrastingly, the transcriptional CDKs are significantly more functionally specialized and conserved from single eukaryotes to multicellular organisms. More complex organisms have larger
numbers, indicating that they further evolved to allow for more complex transcriptional regulation. These roles are summarized in Figure 2.3.

The transcriptional CDKs, including CDKs 7, 9, and 12/13 control transcription through phosphorylation of transcriptional machinery, perhaps most importantly a key regulatory region of RNA Polymerase II, on the carboxyl terminal domain (CTD). The CTD of RNA Pol II consists of 52 heptapeptide repeats of Tyr-Ser-Pro-Thr-Ser-Pro-Ser, which can be phosphorylated on their Tyrosine, Serine, and Threonine changing the affinity of the CTD to transcriptional components and regulating RNA Pol II function.47-49

CDK7/ Cyclin H as part of the general transcription factor TFIIH controls transcriptional initiation and assembly of the pre-initiation complex via phosphorylation CTD of RNA Pol II. Additionally, CDK7 as part of the CAK (CDK Activating Kinase) complex phosphorylates CDKs on their activation T-loops, enhancing cyclin binding.50 CDK9/Cyclin T is part of the P-TEFb (positive transcription elongation factor), and phosphorylates the CTD of RNA Pol II allowing a paused RNA Pol II to begin elongation. P-TEFb binds to the proximal promoter of genes and phosphorylate transcriptional machinery such as NELF (negative elongation factor) as well as the CTD of RNA Pol II to allow for transcriptional elongation.51 Additionally, while less well studied, CDK12 and its paralog CDK13 (bound with Cyclin K) can also phosphorylated the RNA Pol II CTD, however they appear to be most important in regulation of mRNA splicing.48, 52, 53

CDK8/Cyclin C is an important member of these transcriptional kinases, functioning as part of the co-activator complex, Mediator.4, 5, 54, 55 CDK8 function in the context of Mediator has been extensively studied, and new roles for CDK8's function and ability to control transcription of specific transcriptional networks are emerging. CDK8 plays
important structural roles within the context of Mediator, as the deletion of CDK8 or its submodule leads to impairment of transcription.\textsuperscript{54-57} Additionally, deletion of CDK8 is embryonically lethal.\textsuperscript{58} Although it appears CDK8’s kinase activity is more involved with direct phosphorylation of gene specific transcription factors to control their downstream function, either activating or repressing their transcriptional activity.\textsuperscript{7, 11-16, 59-61} Moreover, while core-Mediator is found at active promoter regions, CDK8 is not. Rather CDK8 appears to be enhancer localized and is likely more involved in assisting the transfer of core-Mediator to the promoter proximal regions where it can join with the PIC and allow for transcriptional initiation.\textsuperscript{62}
Figure 2.3: Transcriptional CDKs are highly conserved across eukaryotes and have far more specialized functions. The best studied of these are critical to formation of the pre-initiation complex (PIC) in transcription. CDK7/Cyclin H are part TFIIH, a member of the core transcriptional machinery. CDK8 (CDK19)/Cyclin C are transiently associated with the integral co-activator Mediator and involved in recruitment of transcriptional machinery components, as well as phosphorylation and regulation of gene specific transcription factors. CDK9/Cyclin T are part of PTEFb, the pause control and elongation factor.

2.1.3. Atypical CDKs
While the CDK family has been extensively studied, there are still many members whose biological function is not clearly defined. Most of these belong to the atypical CDKs, whose best studied member is CDK5 (consisting of CDK 10, 14, 15-18, 20 etc). These
CDKs are most structurally related the cell cycle CDKs but tend to have more cell type specific functions.\textsuperscript{63} The best studied of these, CDK5 shows multiple functions including the ability to control pathways integral to neuronal development.\textsuperscript{64} It is likely that as organisms diversified and became more complicated these CDKs were duplicated and developed in a manner to all for more nuanced control of cell type specific functions.\textsuperscript{42}

\subsection*{2.1.4. CDK inhibition and challenges}

CDKs are drivers of many cellular functions and are often mutated or deregulated in cancer.\textsuperscript{65} Thus, making drugs selectively targeting CDKs has been of great interest. Unfortunately, many of the first generation ATP-competitive CDK inhibitors lacked sufficient potency and selectivity to become useful tool compounds and exhibited toxicities that prevented their translational application.\textsuperscript{3} Many early CDK inhibitors, used in pre-clinical and clinical settings, demonstrate significant toxicity likely due to the poor selectivity for individual CDK family members.\textsuperscript{66, 67}

CDKs have been notoriously difficult to target selectively, largely due to the high sequence and shape homology within the CDK family and high degree of active site conservation.\textsuperscript{3, 22} Initial studies assumed development of selective CDK inhibitors would be problematic as most inhibitors (such as flavopirodol, roscovitine, AT7519) had pan-CDK binding.\textsuperscript{3, 22, 65} However, through multiple pharmacological efforts it was shown that selective targeting across the family, though challenging, was achievable.\textsuperscript{3, 65, 68} Currently, various selective CDK inhibitors exist and are in different stages of clinical development for malignancies, including selective CDK4/6 (palbociclib)\textsuperscript{69}, CDK7 (THZ1)\textsuperscript{38}, and CDK9 (NVP2)\textsuperscript{70} inhibitors.\textsuperscript{3, 22, 65, 68, 71} Our lab has developed selective CDK7 (THZ1) and CDK12/13 inhibitors utilizing a Cysteine outside the binding pocket which is not conserved.
across all family members. This allows for a development of more selective covalent inhibitors which irreversibly bind to their targets. As expected, without the pan-CDK toxicity, inhibitors are less toxic allowing for a superior therapeutic index.

However, despite challenges that exist in the CDK family, CDK8 appears to be unique in that it can be selectively with relative ease. Currently, there are multiple published highly selective inhibitors for CDK8, including those which exhibit promising pharmacological profiles.

2.2. Introduction to Mediator, CDK8 and transcription

Mediator is essential for the transcription of almost all protein coding genes in eukaryotes. Mediator consists of a head, middle, and tail domain which allows for multiple docking confirmations of different co-activators or elongation factors. This allows effects on elongation, termination and mRNA processing. Mediator itself exists in two main forms, Core-Mediator, and CDK8-Mediator both of which possess different functions. The Mediator complex regulates transcriptional initiation and assembly of the pre-initiation complex (PIC), presenting a large scaffold to which gene-specific transcription factors (TFs) and general transcription factors (GTFs) bind allowing association with RNA Pol II.

Mediator is found primarily at cis-acting regulatory elements known as enhancers. Recently, it was observed that Mediator together with other transcriptional co-factors and TFs formed unusually large regulatory enhancer regions, known as super enhancers, that regulate genes important to controlling and defining cell identity in both normal and diseased cells.
CDK8 is a transcriptional CDK associated with Mediator. CDK8-Mediator contains four additional subunits that comprise the kinase module: CDK8, CyclinC, Med12, Med13. While loss of CDK8 or its associated subunits is known to be embryonically lethal, the function of CDK8 is only recently become better understood. Initial in vitro studies in yeast and later in mammalian cells suggested that CDK8, and its yeast homolog, Srb10 were capable of phosphorylating the RNA Pol II CTD heptapeptide repeats and preventing PIC assembly. Additionally, in vitro experiments with Mediator and RNA Pol II revealed that the addition of free CDK8/Cyclin C submodule caused PIC disassembly, inhibiting transcription in a dose dependent manner. However, further studies conflicted with the assement of cDK8 as a transcriptional repressor, showing that CDK8 can act as a positive regulator of transcription as well. This discrepancy is highlighted by the ability of CDK8-Mediator to recruit and regulate two CTD kinases; CDK7 as part of the general transcription initiation complex TFIIH as well as CDK9 as part of the transcriptional elongation complex P-TEFb.

In answer to this, work from Jeronimo et al. and Petrenko et al. shows that yeast kinase CDK8 submodule is present at enhancer regions, where it is likely involved in phosphorylation of gene specific transcription factors, but disassociates during the transition of Core-Mediator to the promoter region and PIC. Thus CDK8 submodule may be important for controlling the association of Core-Mediator with enhancer regions. Furthermore, CDK8 kinase activity might initiate transfer of Core-Mediator to promoter regions allowing Core-Mediator to perform its role as a transcriptional co-activator in the PIC. However, following this transfer, the CDK8 submodule does not appear to control
Core-Mediator association with promoter regions in vivo, and in fact cannot be present in the active PIC. This would explain why initial in vitro studies suggested and inhibitory role for CDK8 in purified biochemical systems for transcription.

While CDK8-Mediator does not control basal transcription of genes like Core-Mediator, CDK8 kinase activity is essential in regulating transcription of an established subset of gene networks. In this manner, CDK8 directly phosphorylates multiple transcription factors including S727 of STAT1 (in the transcriptional activation domain), and Ser206 of SMAD1/5 (in the linker region), S375 of E2F1 (inhibiting its β-catenin inhibitor activity), basal phosphorylation of S727 of STAT1 in NK cells, Notch1 signaling in TAL, as well as NFKB dependent innate immune activation. Thus CDK8 phosphorylates transcription factors to control their activity, elucidating the importance of CDK8 kinase activity in transcriptional regulation.

2.3. CDK8 roles in disease and cancer
Numerous lines of evidence suggest that CDK8 may be a viable drug target. CDK8 is highly up-regulated in multiple cancers, particularly in cells of gastroinsteional (GI) lineages. CDK8 overexpression is detected in 70% of patient tumor samples in colon cancer and is linked with β-catenin hyperactivity. Mutations effecting β-catenin stability, often through perturbed regulatory APC binding, have long been linked to tumors of GI lineage. Increased CDK8 expression is tightly correlated with poor overall patient prognosis. Consistent with these findings, knockdown of CDK8 leads to an inhibition of cellular proliferation and tumorigenic potential. Interestingly, this loss of CDK8 was correlated with a decrease in MYC and MYC target genes.
The tumorigenic effect of CDK8 appears directly linked to its kinase activity. While induction with CDK8 causes transformation immortal murine fibroblasts (NIH 3T3 cells), the kinase dead variant has no such capabilities. Moreover, transfection of kinase dead CDK8 into transformed cells causes cell death. Additional studies have corroborated these findings, indicating that CDK8 kinase activity is a major regulator of colon cancer and stem cell function leading to tumor de-differentiation in vivo.

CDK8's tumorigenicity is not limited to colon cancer. In gastric adenocarcinoma, CDK8 silencing decreased proliferation, while CDK8 activation promoted tumor progression in vivo. Additionally, increased CDK8 expression is correlated with tumor grade in patient samples. Likewise, CDK8 silencing in melanoma caused decreased proliferation while activation promoted progression. This may be due to the importance of the Wnt/β-catenin pathway in melanoma. Furthermore, loss of CDK8 in breast cancer inhibits proliferation, colony formation, migration and may have G0/G1 cell cycle arresting effects. However, a recent publication revealed that CDK8's tumor promoting effects may differ based on tumor type or genetic history. CDK8 appears to act more as a tumor suppressor in endometrial cancers, following the observation of decreased proliferation with CDK8 overexpression and increased proliferation with CDK8 knockdown. Additionally, in TAL cancers, CDK8's binding partner Cyclin C appears to act instead as a tumor suppressor.

Beyond the direct oncogenic potential of CDK8, there are additional biological roles for CDK8. Recently, the CDK8 drive phosphorylation of pS727 mark on STAT1 was shown to lead to down-regulation of natural killer cell tumor surveillance and cytotoxic potential. Thus selectively inhibiting CDK8 could potentially act as an immune activating
agent. Also, CDK8 has been implicated as an intermediate in p21-mediated chemotherapy induced tumor promoting paracrine activity. DNA damaging and cytotoxic agents may lead to cell death in the tumors but can also induce a protective effect via secreted paracrine factors from surrounding healthy tissue. This activity is thought to be modulated through p21 signaling via CDK8. This indicates inhibition of CDK8 is a potential strategy for therapeutic intervention via multiple pathways.

2.4. Small molecule inhibitors of CDK8

2.4.1. Natural Products; The Cortistatins

The Cortistatins are a family of steroidal alkaloids initially isolated from the sea sponge *Corticium simplex*. These compounds were of interest as they showed potent anti-proliferative activity against human umbilical vein endothelial cells (HUVECs), the most potent of these being Cortistatin A. A close analog, didehydrocortistatin A (DCA) was shown to have selective binding for only one member of the CDK family, CDK8 and its paralog CDK19—as well as an unrelated kinase ROCK1. Additional work indicated the Cortistatin may have activity on HIV Tat dependent transcription. However, due to its poor availability with a complicated total synthesis, it has not been widely used in study of CDK8 biology.

A recent paper by the Shair lab, identified a subset of AML cell lines which were sensitive to CDK8 inhibition with Cortistatin A. Interestingly, when looking at the impact of CDK8 inhibition on the transcriptional landscape of these cells, they saw an increase in transcription of a subset of super enhancer like genes. This implied that CDK8 was negatively regulating super enhancers in these AML cells. This is in direct contrast to other transcriptional CDKs such as CDK7 which are integral to the expression of most
super enhancers, and whose inhibition significantly decreases their expression.\textsuperscript{38} Additionally, Cortistatin A showed good pharmacokinetic properties and promising efficacy in mouse xenograft models of the sensitive AML lines. Thus, despite the challenging total synthesis of the Cortistatin family, it may yet prove to be a promising therapeutic moiety for the \textit{in vivo} inhibition of CDK8.

\textbf{2.4.2. Senexin A}

Traditional chemotherapy and radiation can result in off target effects on surrounding tissues. While the therapy can lead to the death of the tumor cells, it can also result in changes in the tumor microenvironment which prevent full treatment efficacy. This comes about as the toxic therapies lead to tumor promoting paracrine activity in the surrounding non-cancerous cells, leading to angiogenesis, tumor resistance, and tumor promoting cytokines.\textsuperscript{95, 106}

Much of this is regulated via p53/p21 signaling axis for recognizing DNA damage and cellular stress. Thus Porter et al. endeavored to find small molecules that regulate the p53/p21 axis. They identified a small molecule, Senexin A, which was capable of regulating p21 and NFKB transcriptional activity in response to induction with chemotherapy treatments, with minimal effect on basal level transcription.\textsuperscript{95} Further experiments showed that p21 bound to and activated CDK8 kinase activity. Additionally, this small molecule selectively inhibited CDK8 and CDK19 across the kinome and, more intriguingly, across the CDK family. This proved to be the first CDK8 inhibitor identified \textit{de novo} from a pathway specific screen. While it is far less potent than the Cortistatin family, discovery of Senexin A furthered the possibility that discovery of a selective CDK8 inhibitor with increased synthetic tractability was possible.
2.4.3. CCT Compounds from Merck

More recently a group of CDK8 inhibitors has been reported from groups affiliated with Merck, Cancer UK, and University of Cardiff, after identifying a small molecule which regulates WNT dependent signaling. Following a target identification effort, they determined the small molecule CCT251545 which potentially inhibited CDK8 and CDK19 in the single digit nano-molar region, with reasonable selectivity, around 100X over off-target kinases. Additionally, CCT251545 successfully inhibited established marks of CDK8 activity, including phosphorylation of S727 of STAT1 following IFNγ induction. CCT251545 also showed reasonable in vivo efficacy on WNT dependent tumors.

Following this, the group endeavored to design additional small molecule probes and drugs for CDK8, alighting on multiple scaffolds with varying pharmacological activity, including a scaffold based of a HSP90 inhibitor, as well as an orally bioavailable small molecule CCT251921 which showed properties promising for continuing into small molecule cancer models. These small molecules showed good selectivity, with minimal off target kinase inhibition.

However, once progressing into in vivo studies with these scaffolds, some troubling toxicities became evident. While the drug treatment in vivo showed evidence of target engagement via monitoring of WNT dependent transcription, there was only modest activity against patient derived-xenografts. Thus, none of the compounds were tolerated at the high doses needed for efficacy in tumor models. Moreover, the toxicities observed were relatively consistent between the two of the structurally different CDK8 inhibitor scaffolds, likely indicating that the toxicity was due to on-target inhibition of CDK8. This body of works suggests that inhibition of CDK8 may have limited functionality as a cancer therapeutic, as the high doses required for efficacy also lead to toxicity. However, with
emerging evidence for CDK8’s role in regulation of immune activity, it does not eliminate CDK8 as a therapeutic target across all diseases, where lower amounts of drug may be capable of achieving the desired phenotype.
Chapter 3: Exploring effect of CDK8 inhibition on transcription
3.1. Abstract

CDK8 is a transcriptional CDK transiently associated with the multidomain protein complex, Mediator, which is a transcriptional co-activator exclusively found in eukaryotic cells. Within CDK8-Mediator, CDK8, CyclinC, Med12, and Med13 comprise the four subunit kinase module. CDK8 is essential for preimplantation development in mice; total loss of CDK8 resulted in fragmented blastomers and early embryonic lethality. Presence of CDK8-Mediator is integral in transcription of specific gene networks, as well as for recruitment of certain essential transcription and elongation factors. However, it is not clear whether the kinase activity is indispensable for CDK8’s function within Mediator. Selective protein kinase inhibitors have proven integral in elucidating kinase function in cellular signaling pathways. The temporal precision of pharmacological CDK kinase inhibitors presents a complementary approach relative to RNAi mediated depletion, which take longer to manifest, and may elicit phenotypes derived from loss of scaffolding function as opposed to exclusive loss of kinase activity. Dissection of CDK8 function using a CDK8 specific chemical probe would allow for a comprehensive understanding of the importance of CDK8 kinase function.

3.2. Introduction

3.2.1. CDK8 in Colorectal Cancer

Firestien et al. identified a compelling role for CDK8 in its ability to regulated beta catenin dependent gene expression in colorectal cancer cells. Colorectal cancer cells commonly exhibit upregulation of β-catenin signaling, often in concert with kRas mutations. When present, these mutations make colorectal cancers highly aggressive and challenging to treat with current therapeutic strategies. Thus Firestien et al. utilized a
siRNA screen to identify targets which when knocked down limited β-catenin signaling, identifying CDK8. Additionally, cells which co-expressed high levels of CDK8 and high levels of β-catenin were most sensitive to CDK8 knockdown. Moreover, CDK8 was transformative in these cells, but only while it maintained its kinase activity, as catalytically dead kinase was not transformative. This work made CDK8 appear to be a potentially promising oncogenic target in colorectal cancer cells.8, 9

3.2.2. Discovery of CDK8 as a regulator of STAT1 phosphorylation

The JAK/STAT axis is important in multiple biological processes, most especially in immune regulation. Canonically, Jak family proteins phosphorylate STAT (Signal Transducer Activator of Transcription family of proteins) on a conserved activating tyrosine residue, leading to its dimerization to an identical STAT (homo-dimerization) or related family member (hetero-dimerization), followed by nuclear translocation and binding to the STAT sites and activation of transcription.108 While canonical STAT driven transcription requires tyrosine phosphorylation, which allows for nuclear translocation, there are additional residues which regulated STAT activity. This includes a regulatory serine in the transactivation domain (TAD) which controls the duration and amount of STAT dependent genes which are transcribed.109 STAT1 TAD Serine 727 is known to be phosphorylated by the MAPK family following exposure to various Toll-Like Receptor (TLR) ligands or under cellular stress to serve as a “priming” of STAT activity, before tyrosine phosphorylation.11 However, following IFNγ induction, JAK phosphorylates STAT1 on the tyrosine, and an different kinase phosphorylates STAT1 in its TAD. In 2013 CDK8 was identified as responsible for phosphorylating STAT1 S727 following IFNγ
induction, a result which has been consistently used in later research as a biomarker for CDK8 target engagement. 11, 91

3.3. Results

3.3.1. Selectivity and Efficacy of DCA
Didehydrocortistatin A (Figure 3.1a) was profiled using the high-throughput biochemical screen of kinase binding (KINOMEscan; Ambit Biosciences, San Diego, CA) revealing single digit nanomolar binding for one member of the CDK family, CDK8, and exquisite selectivity across the kinome. Significant additional binding was only also observed for ROCK1 and ROCK2, and did not appear to be evident in subsequent studies. 72 However while this assay gives insight to small molecule binding across the recombinantly expressed kinases, there are limitations. This assay only identifies competitive binders and does not guarantee those binders will translate to catalytic inhibition. Moreover, the in vitro expressed kinases are not always faithful mimics of cellular activity. Additionally, the profiling work was completed on Cortistatin A which shows similar but not identical structure and activity to DCA (Figure 3.1). DCA itself has not been extensively studied.

Following establishment of DCA as a CDK8 kinase inhibitor, DCA was profiled using the colorectal cancer cell line HCT116 using the KiNativ™ assay. 110 This assay uses a desthiobiotin modified ATP or ADP which non-specifically labels ATP dependent enzymes. However, small molecule binding elicits a protective effect, reducing labeling. The total cohort of labeled enzymes are enriched via streptavidin pull down and detected via mass spectrometric analysis against a library of established peptide fragments. This allows for a comprehensive analysis of the kinases which DCA binds in a cellular context.
DCA treatment resulted in a significant decrease in detection of CDK8 related peptides but had little impact on the detection of other kinases as shown in Figure 3.1b. Additionally, there was no detected binding to any other members of the CDK family or ROCK1/2. This suggests a high level of selectivity for DCA, which has been challenging to achieve for chemical probes of the CDK family.
Figure 3.1: Summary of biochemical profiling and subsequent selectivity of the DCA. a) The structure of Cortistatin A, and Didehydrocortistatin A (DCA). b) KINOMEscan; (Ambit Biosciences, San Diego, CA) profiling showing that DCA only appears to bind CDK8/Cycling C with any high degree across all kinases profiled.
3.3.2. Anti-Proliferative Effect on Colorectal Cancer Cells
Following the work done by Firestien et. Al.\textsuperscript{7-9}, DCA was tested on a panel of colorectal cancer cell lines which were previously established as sensitive to CDK8 knock down and dependent on β-catenin/Wnt signaling. Firestein et al. suggested that CDK8 kinase activity was integral for the transformative activity, thus CDK8 would be an ideal therapeutic target for inhibition. However, none of the tested cell lines displayed any sensitivity up to 10µM concentrations of DCA, which is 10,000X above the biochemical inhibitory concentration of the DCA. Thus, CDK8 inhibition did not affect cellular viability of these colorectal cancer cells (Figure 3.2).
Figure 3.2: Cell viability results from testing the CDK8 inhibitor DCA colorectal cancer lines purportedly sensitive to CDK8 knock down via siRNA. DCA was tested on 5 colorectal cancer cell lines (COLO205, SW480, HT29, T84, COLO741) all reported to have varying dependence on CDK8 in Firestien et al. However, all lines were determined to be completely insensitive to DCA treatment, suggesting that while CDK8 knock down may elicit an anti-proliferative effect, CDK8 inhibition does not have the same impact.
3.3.3. CDK8 inhibition regulates a subset of genes in IFNγ induction

Initial studies were done using IFNγ responsive HepG2 cells. IFNγ stimulation activates the JAK/STAT pathway resulting in phosphorylation of STAT1 at two residues; Y701—necessary for STAT1 transcriptional activity—and S727 (in the transactivation domain)—necessary for full expression of IFNγ responsive gene. JAK is the established tyrosine kinase responsible for phosphorylation Y701 of STAT1, however CDK8 was recently established as the kinase responsible for the phosphorylation of STAT1 at S727 following IFNγ stimulation. Western blotting was used to confirm phosphorylation of the STAT1 residues and show that DCA is capable of inhibiting pSTAT1 S727 following IFNγ induction, as shown in Figure 3.3, down to as low as 10 nM concentration of DCA, with no impact on the JAK induced pY701. This assay proved to be a robust method to measure a selective cellular inhibition of CDK8 activity by monitoring of the pSTAT1 S727 mark.
Figure 3.3: Inhibition of phosphorylation of a known cellular target of CDK8. Treatment of HepG2 liver carcinoma cells with DCA from a high concentration of 1 µM to a low concentration of 10 nM shows a dose dependent inhibition of CDK8 driven phosphorylation of STAT1 S727 following IFNγ stimulation.
I examined the changes in global RNA expression using GeneChip® PrimeView™ Human Gene Expression Array to identify the transcriptional changes that occur following CDK8 kinases inhibition. This was explored in HepG2 cells treated with DCA to determine genes are dependent upon CDK8 kinase activity for their transcription, and compared to HepG2 cells stimulated with IFNγ following DCA treatment to determine IFNγ dependent genes reliant on CDK8 kinase activity for transcription. Gene expression was normalized using RNA spike in control methods established in the Young lab and data analysis was done with the help of the Young lab.

The global analysis, as seen in Figure 3.4a, indicated that there was minimal impact on global gene expression following DCA treatment. However, further analysis showed CDK8 inhibition selectively regulated a subset of genes most affect following IFNγ stimulation Figure 3.4b. As seen in Figure 3.4b, the genes (in blue) which are most highly activated upon IFNγ stimulation have blunted activation when treated with DCA. Additionally, the genes that are normally repressed (in red) following IFNγ stimulation, are now slightly de-repressed. The most strongly impacted transcripts identified from the microarray data were validated via qPCR (Figure 3.4c). STAT1 and IL15 transcripts were selectively repressed with CDK8 inhibition following IFNγ stimulation.
Figure 3.4: Changes in gene expression following CDK8 inhibition under conditions of IFNγ were determined using GeneChip® PrimeView™ Human Gene Expression Array. a) Cells were pre-treated with 50nM or 100nM of DCA followed by stimulation with or without IFNγ. From there, gene expression was compared to DMSO treated control cells. Again, there is minimal observed changes in gene expression as shown in the heat map. b) Box and whisker plots showing changes in expression of top induced (blue), repressed (red), and random (green) genes. Without IFNγ, there is again minimal changes. However, DCA/+IFNγ compared to DMSO/+IFNγ condition, have clear differences. Induction (blue) of genes by IFNγ is blunted with DCA, and repression (red) of genes is de-repressed, while there is no observable change globally (green). This suggests that CDK8 activity controls a subset of genes following IFNγ stimulation. c) Confirmation of this in both HepG2 and Jurkat cells using qPCR shows that both STAT1 and IL15 expression is specifically repressed by CDK8 activity only following IFNγ stimulation.
3.4. Discussion:
These initial studies established whether the kinase activity of CDK8 is required for CDK8 to regulate transcription, specifically in the context of IFNγ dependent genes. More work will be required to understand the detailed mechanism of action of transcriptional modulation via CDK8 kinase inhibition. Already, I have seen different levels of sensitivity of IFNγ gene sets to CDK8 inhibition.

Moreover, while CDK8 has been established as the regulator of IFNγ dependent STAT1 phosphorylation in the transactivation domain, it has also been suggested to phosphorylate STAT1 independently of stimulation in NK cells. These NK cells exhibit constitutive phosphorylation of STAT1 at S727 contained in the transactivation domain without the associated JAK induced Tyr701 phosphorylation mark that is found following IFNγ stimulation.

Interestingly, this constitutive phosphorylation is associated with decreased tumor surveillance capabilities and is modulated via CDK8. Thus, once establishing the importance of CDK8 kinase activity in IFNγ stimulated pSTAT1 S727, it may be possible to examine the impact of CDK8 inhibition in NK cells as an immune modulating therapy. While CDK8 was proposed as a promising oncogenic target, initial work in our lab indicated that there is a divergence between the role that CDK8 has structurally, where its presence is necessary for the recruitment of general transcriptional machinery, as compared the role of its kinase activity.

While CDK8 knock down shows an anti-proliferative effect, inhibition of its kinase activity does not mimic this phenotype. However, CDK8 kinase activity does play a clear role in regulation of gene specific transcription factors, such as STAT1, via direct
phosphorylation. While it remains to be seen if CDK8 inhibition is a valid target for oncogenic therapy, it may be ideal for controlling cellular responses to external stimuli.

3.5. Materials and Methods

**Cell viability assays.** Colorectal cancer cell lines were grown according to ATCC® guidelines. COLO205 RPMI1640; SW480 DMEM, HT29 McCoy’s 5a, T84 DMEM/F12, COLO741 DMEM supplemented with 10% FBS and pen/strep. Cells were grown to 70% confluence in T75 flasks, then trypsined and plated at 5000 cells/well. Compounds were added in dose, and allowed to sit for 72hrs. Cellular viability was measured using Cell Titer Glo™ according to the manufactures protocol.

**Western Blotting.** HepG2 cells were grown in DMEM with 10% FBS. Cells were seeded in 6 well dishes at 2.5X10^6 cells per well. Cells were pre-treated with DCA in dose, then stimulated with 50ng/mL of human IFNγ (Peprotech™) for 1 hour. Cells were harvested by scraping in cold PBS then lysed using RIPA (Invtrogen™). Protein was normalized using BCA kit (Pierce Biosciences), then boiled with LiDS sample buffer for 5 minutes at 95C. Samples were chilled on ice 10 minutes, then run on at 4-12% acrylamide gel, transferred to a nitrocellulose membrane, blocked in BSA, then blotted for STAT1 and pSTAT1 S727 and pSTAT Y701 using antibodies available through Cell signaling technologies.

**Gene expression.** HepG2 cells were seed in a 6 well dish at 2.5X10^6 cells per well, and treated in the same was as for western. Cells were then harvested using TRIzol reagent, and RNA purified using miRNeasy kit from Invitrogen. Purified RNA concentration was measured using nanodrop. Global mRNA expression was determined using gene chip arrays using GeneChip® PrimeView™ Human Gene Expression Array at the Whitehead Institute.
sequencing core, and analyzed with assistance from Brian Abrahams from Rick Young's lab at Whitehead and MIT.

**Quantitative Real-Time PCR.** TaqMan Primer/Probe sets for IL15 and STAT1 were purchased from Invitrogen, and TaqMan® Master Mix (Invitrogen). qPCR reactions were run in a 96 well format on QuantStudios™ 6 RT-PCR System.
Chapter 4: Identification and Investigation of Small molecule regulators of the innate immune system
4.1. Development of Chemical Probes for Investigation of Salt-Inducible Kinase Function In Vivo

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The manuscript below, published in ACS Chemical Biology in August of 2016 describing efforts to understand the pharmacology and biology of Salt-Inducible Kinase inhibitors synthesized by Yanke Liang, and primarily profiled by Thomas Sundberg. The SIK inhibitors showed initial promise as potential small molecule therapeutics for Inflammatory Bowel disease, via their ability to promote tolerance in Myeloid cells and increase production of IL-10. I aided in profiling these inhibitors and determining their effect on IL-10 transcription and cytokine production (See Figure 4.2 c,d), and provided edits on the manuscript during the review process. The paper is as follows:
4.1.1. Abstract
Salt-inducible kinases (SIKs) are promising therapeutic targets for modulating cytokine responses during innate immune activation. The study of SIK inhibition in animal models of disease has been limited by the lack of selective small-molecule probes suitable for modulating SIK function in vivo. We used the pan-SIK inhibitor HG-9-91-01 as a starting point to develop improved analogs, yielding a novel probe 4 (YKL-05-099) that displays increased selectivity for SIKs versus other kinases and enhanced pharmacokinetic properties. Well-tolerated doses of YKL-05-099 achieve free serum concentrations above its IC\textsubscript{50} for SIK2 inhibition for > 16 hours and reduce phosphorylation of a known SIK substrate in vivo. While in vivo active doses of YKL-05-099 recapitulate the effects of SIK inhibition on inflammatory cytokine responses, they did not induce metabolic abnormalities observed in Sik2 knockout mice. These results identify YKL-05-099 as a useful probe to investigate SIK function in vivo, and further support the development of SIK inhibitors for treatment of inflammatory disorders.

4.1.2. Main Text
Salt-inducible kinases (SIK) 1–3 are serine/threonine kinases in the adenosine monophosphate-activated protein kinase (AMPK) family first recognized for their role in energy metabolism where they link G protein-coupled receptor (GPCR)/cAMP signaling to gene expression programs that increase gluconeogenesis in hepatocytes and regulate lipid metabolism in adipose tissue\textsuperscript{117-120}. Under basal conditions, SIKs phosphorylate the CREB-regulated transcriptional coactivators (CRTC) and class IIa histone deacetylases (HDAC4, 5, 7 and 9), resulting in their cytosolic sequestration by phosphorylation-dependent interactions with 14-3-3 proteins\textsuperscript{120, 121}. Inhibitory phosphorylation of SIKs by
protein kinase A (PKA) in response to elevated intracellular cAMP enables CRTC in response to elevated intracellular cAMP enables CRTC and class IIa HDACs to enter the nucleus and coordinately regulate gene expression

As such, SIKs are critical mediators of signaling induced by hormones like glucagon or catecholamine that activate GPCRs in metabolic tissues.

More recently, SIKs have been identified as key regulators of GPCR-modulated cytokine responses in innate immune cells like macrophages and dendritic cells. For instance, PKA-dependent suppression of SIK activity is observed in innate immune cells treated with the prostanoid receptor agonist prostaglandin E2 (PGE2)\textsuperscript{125, 126}. Inhibiting SIK activity converts innate immune cells to a more tolerogenic state characterized by increased CREB-dependent expression of the anti-inflammatory cytokine interleukin-10 (IL-10), as well as reduced inflammatory cytokine expression due to deacetylation of NF-κB subunits by class IIa HDACs\textsuperscript{125-127}. Of note, directly targeting SIKs with small-molecule inhibitors recapitulates many of the immunomodulatory effects induced by elevated intracellular cAMP\textsuperscript{17, 128}.

The physiological role of SIKs has been studied in knockout mice. Sik3\textsuperscript{-/-} mice are born below Mendelian ratios and survivors display a malnourished phenotype, insulin handling defects, and increased sensitivity to lipopolysaccharide (LPS)-induced production of IL-6 and death\textsuperscript{129, 130}. In contrast, Sik2 knockout mice are grossly normal, but have enlarged fat cells, increased macrophage infiltration of adipose tissue, hypertriglyceridemia and decreased plasma adiponectin levels\textsuperscript{119}. Consistent with the role of adiponectin in promoting glucose utilization, Sik2\textsuperscript{-/-} mice display modestly elevated blood glucose levels following glucose or insulin challenge\textsuperscript{119}. Although these studies provide insight into the physiological roles of SIKs, acutely inhibiting SIK activity with small
molecules may not recapitulate the developmental and metabolic defects associated with
genetic deletion of these kinases. To address this question, we developed SIK-targeting
small-molecule inhibitors with pharmacokinetic (PK) properties suitable for use in vivo,
and applied these tools to study the impact of acute SIK inhibition on specific
immunomodulatory and metabolic responses regulated by these kinases.

We first determined if the SIK-targeting cell-based inhibitor HG-9-91-01 has PK
properties amenable for use in vivo. HG-9-91-01 was > 99% serum bound and rapidly
degraded by mouse liver microsomes (t1/2 = 11 min) making this compound unsuitable for
direct injection into animals. To guide efforts to develop HG-9-91-01 derivatives with
improved PK properties, we docked the inhibitor to a homology model built using the
available crystal structure of MARK3/Par-1 kinase domain (PDB ID: 3FE3) (Figure 4.1A),
which has 81% sequence similarity to SIK2 kinase domain131. This model predicts that
the 4,6-diaminopyrimidine core of HG-9-91-01 forms a pseudo-bicyclic ring that orients
the urea carbonyl to interact with Lys-49; this finding suggests that replacing the pseudo-
bicyclic ring with a fused pyrimidopyrimidinone core (e.g., 1; Table 4.1), a modification
expected to improve microsomal stability, might be tolerated. While 1 exhibits improved
microsomal stability (t1/2 = 20 min) and retains the ability to inhibit SIK2 and enhance IL-
10 production by activated bone marrow-derived dendritic cells (BMDCs), it is highly toxic
in this cell type. Substitution of the 2,4-dimethoxy-phenyl side chain with a 3-
methoxypyridine group further increased microsomal stability (t1/2 = 27 min for 2), but
again resulted in significantly elevated toxicity. These results suggest the need to
incorporate additional modifications to mitigate the toxicity observed with the more stable
fused pyrimidopyrimidinone core.
Figure 4.1: Molecular modeling informs development of SIK inhibitors with improved pharmacokinetic properties. A) HG-9-91-01 docked into the kinase domain of MARK3/Par-1 kinase. B) Chemical structure and summary of structure-activity relationship data for development of YKL-05-099. C) The activity of 141 kinases were measured in the presence of 3 (1 μM) or YKL-05-099 (1 μM) and is reported as the percent untreated activity that remains in the presence of the inhibitor (mean, n = 2 from 1 independent experiment). Table displays kinases inhibited by YKL-05-099 (1 μM) with < 10% activity remaining (mean, n = 2 from 1 independent experiment).

As HG-9-91-01 and its derivatives are potent inhibitors of a number of kinases including many members of the Src-family kinases, we hypothesized that improving the kinase selectivity towards SIKs might reduce the cellular toxicity. To accomplish this we explored substitution of 2-anilino substituent, which is well known to modulate kinase selectivity in the context of structurally similar kinase inhibitors. Methyl ether substitution of the ortho position yielded 3, a promising analog that retains potent SIK2-inhibitory (IC$_{50}$ = 34 ± 14 nM) and IL-10-enhancing (EC$_{50}$ = 70 ± 40 nM) activities while only displaying cell-based toxicity at concentrations > 5 μM (Table 1). Ethyl or isopropyl
ether substitution of the ortho position of the 2-anilino substituent progressively impaired SIK2-inhibitory activity (Table 4.1), which suggests that this site may be sterically limited in SIK2. Hence, methyl ether substitution of the aniline tail appears to effectively maintain potent SIK2-inhibitory and IL-10-potentiating activities, while mitigating toxicity associated with the fused core.

Table 4.1: Activity profile and microsomal stability of HG-9-91-01 derivatives. Inhibition of SIK2 (IC\textsubscript{50}) as well as effects on IL-10 production (EC\textsubscript{50}) and cellular cytotoxicity (CC\textsubscript{50}) of Zymosan A-stimulated BMDCs are presented as the mean ± SD from ≥ 2 independent experiments. Mouse liver microsome (MLM) t\textsubscript{1/2}'s are from 1 independent experiment. N.D., not determined.

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<th>R1</th>
<th>R2</th>
<th>R3</th>
<th>SIK2 IC\textsubscript{50} (nM)</th>
<th>IL-10 EC\textsubscript{50} (nM)</th>
<th>Tox IC\textsubscript{50} (\mu M)</th>
<th>MLM t\textsubscript{1/2} (min)</th>
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<td>3 ± 2</td>
<td>0.007 ± 0.005</td>
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<tr>
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<td>Cl</td>
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<td>6 ± 3</td>
<td>11 ± 1</td>
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<tr>
<td>3</td>
<td>Cl</td>
<td>Cl</td>
<td>N</td>
<td>34 ± 14</td>
<td>70 ± 40</td>
<td>6 ± 1</td>
<td>37</td>
</tr>
<tr>
<td>4 (YKL-05-099)</td>
<td>Cl</td>
<td>Cl</td>
<td>N</td>
<td>40 ± 25</td>
<td>460 ± 110</td>
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<td>N.D.</td>
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Converting the terminus of the 2-aniline substituent to a 1-methylpiperidine group yielded YKL-05-099 (Figure 4.1B), which has slightly less potent SIK2-inhibitory (IC\textsubscript{50} = 0 ± 25 nM) and IL-10-enhancing activities (EC\textsubscript{50} = 460 ± 110 nM), but is non-toxic at concentrations ≤ 10 μM and stable in mouse liver microsomes for > 2 hr (Table 4.1). In addition, YKL-05-099 is highly soluble (PBS solubility = 428 ± 11 μM) and present in an
unbound state at appreciable levels in mouse plasma (Free fraction = 6 ± 1%). Consistent
with the observations for ceritinib, methyl ether substitution of 4’s aniline tail improved
kinase selectivity for SIK2 and 3 relative to the unsubstituted analog YKL-05-095 (Figure
4.1C), and overall selectivity against a panel of 468 kinases. In addition, we confirmed
that 4 binds to SIK1 and SIK3 with IC₅₀’s ~10 and ~30 nM, respectively in a competitive
LanthaScreen® Eu kinase binding assay. The activity profile, in vitro PK properties, and
improved SIK kinase selectivity highlights YKL-05-099 as a promising probe for exploring
the functional consequences of SIK inhibition in cells and in vivo.

We next determined whether, in addition to up-regulating IL-10 (Figure 4.2A),
YKL-05-099 induces cell-based phenotypes consistent with SIK inhibition. First, we found
that pre-incubating bone marrow-derived macrophages (BMDMs) with YKL-05-099
reduced LPS stimulated phosphorylation of HDAC5 at the SIK-specific phosphorylation
site Ser259₁²₃, ₁²₄ (Figure 4.2B). Given that potentiation of CREB activity is a known
consequence of inhibiting SIKs in many cell types ₁⁷, ₁₁₈, ₁²₅, ₁₂₈, ₁₃₃, we asked whether YKL-
05-099 up-regulates expression of the CREB target genes Il10 and Nurr77 ₁²₅. Indeed,
levels of both transcripts were increased by pre-incubation of BMDCs with YKL-05-099
prior to LPS stimulation (Figure 4.2B). Finally, as observed for other SIK inhibitors ₁⁷, ₁₂₈,
YKL-05-099 suppressed production of the inflammatory cytokines TNFα, IL-6 and IL-
12p40, and only modestly enhanced IL-1β release in BMDCs stimulated with the yeast
cell wall extract Zymosan A (Figure 4.2D). Collectively, these data indicate that YKL-05-
099 affects responses previously linked to SIK inhibition in activated innate immune cells.
Figure 4.2: 4 displays cell-based activities consistent with SIK inhibition. A) Pre-treatment with the indicated concentrations of YKL-05-099 for 24 hr potentiates IL-10 production by Zymosan A-stimulated BMDCs (mean ± SD; n = 3 from 1 independent experiment; Quantified as % of the response elicited by PGE2). B) Pre-incubation of BMDMs with YKL-05-099 (1 μM) or HG-9-91-01 (1 μM) for 6 hr prior to stimulation with LPS for 30 min reduces p-HDAC5 (Ser259) levels. Ratio of p-HDAC5 / HDAC5 quantified as mean ± SD, n = 2 from 1 independent experiment. C) Effect of pre-treatment with YKL-05-099 for 24 hr on Il10 and Nurr77 transcripts in BMDCs stimulated with LPS for the indicated time points (mean ± SD, n = 6 from 1 independent experiment). D) Pre-incubation of BMDCs with YKL-05-099 (1 μM) for 24 hr modulates inflammatory cytokine production induced by stimulation with Zymosan A for 18 hr (mean ± SD, n = 3 from 1 independent experiment). *** P < 0.001; ** P < 0.01 using unpaired Student’s t tests. All data are representative of ≥ 2 independent experiments.

Given that YKL-05-099 has biochemical and cell-based activities consistent with SIK inhibition and favorable in vitro PK properties, we next asked whether well-tolerated doses of YKL-05-099 achieve free serum concentrations sufficient to inhibit SIKs in vivo. Following IP administration at 20 mg/Kg, YKL-05-099 is rapidly absorbed followed by slow clearance (t_{1/2} > 7 hr) such that serum concentrations exceeding the IC_50 for SIK2 inhibition are maintained for > 16 hr. Of note, no obvious changes in viability, activity or grooming were induced by YKL-05-099 during the 24 hr treatment period. To demonstrate
SIK engagement in vivo, we pre-treated mice with 5 – 50 mg/Kg 4 for 15 min prior to stimulation with LPS for 1 hr, and then generated lysates from total splenic leukocytes. In this experiment, YKL-05-099 dose dependently decreased phosphorylation HDAC5 at the SIK-regulated site Ser259; reduced phosphorylation was observed at the lowest dose (5 mg/Kg) and was below the limit of detection by immunoblotting beginning at the 20 mg/Kg dose (Figure 4.3A). While total HDAC5 levels are reduced by the higher doses of YKL-05-099, normalizing the change in HDAC5 phosphorylation at Ser259 to total HDAC5 confirms the YKL-05-099-induced decrease in this phosphorylation event (Figure 4.4). These data suggest that well tolerated doses of YKL-05-099 are able to inhibit SIKs in vivo.
Figure 4.3: YKL-05-099 modulates inflammatory cytokine responses in vivo. A) and B) IP administration of the indicated doses of YKL-05-099 for 15 min prior to stimulation with LPS (0.5 mg/Kg) for 1 hr reduces p-HDAC5 (Ser259) levels in total splenic leukocytes and modulates serum IL-10 and TNFα levels (bars = mean; n = 3 mice; data is representative of 2 independent experiments). ***, P < 0.001; *, P < 0.05 using two-way ANOVA with Dunnett post-test. C) and D) IP administration of YKL-05-099 (20 mg/Kg) for 15 min prior to stimulation with LPS (0.5 mg/Kg) modulates serum IL-10 and TNFα abundance and colonic mRNA levels without affecting IL-6 responses (mean ± SD; n = 3 mice per time point; data is from 1 independent experiment). ***, P < 0.001; **, P < 0.05; *, P < 0.05 using unpaired Student’s t tests.
Supplementary Figure 4.4: Evidence that YKL-05-099 engages SIKs in vivo. A) Free serum concentrations of YKL-05-099 exceed the IC<sub>50</sub> for SIK2 inhibition (40 ± 25 nM) for > 16 hr following IP administration of 20 mg/Kg in C57BL/6 mice (mean, n = 3 mice per time point, data is from 1 independent experiment). B) Reduction in p-HDAC5 (Ser259) in splenic lysates from YKL-05-099 treated mice quantified relative to total HDAC5 levels (mean ± SD; n = 3 mice; data is representative of 2 independent experiments). **, P < 0.01 based on two-way ANOVA with Dunnett post-test.

To determine whether SIK inhibition affects systemic inflammatory responses in vivo, we quantified serum IL-10 and TNFα levels from mice briefly pre-treated with YKL-05-099 prior to stimulation with LPS for 1 hr. Consistent with the cellular consequences of SIK inhibition, YKL-05-099 dose-dependently reduced abundance of TNFα in serum beginning at 5 mg/Kg, and increased IL-10 levels at the 20 mg/Kg dose by > 2-fold (Figure 4.3B). The differential effects of 5 mg/Kg YKL-05-099 on TNFα suppression versus IL-10 potentiation may reflect different sensitivities of class IIa HDACs versus CRTC proteins to SIK inhibition. In a separate experiment, we found that YKL-05-099 up-regulated IL-10 and reduced TNFα in serum from LPS-stimulated mice for up to 6 hours, suggesting its immunomodulatory effects are sustained over time (Figure 4.3C). Of note, treatment with YKL-05-099 moderately decreased serum IL-6 levels (Figure 4.3C), which contrasts with the reported effects of LPS-stimulation in Sik3 KO mice<sup>130</sup>. This contrasting phenotype may reflect the difference between pan-SIK inhibition by YKL-05-099 in our experiment versus Sik3-specific deletion in the genetic knockout model in which SIK1 and 2 may compensate for loss of SIK3. In addition to serum measurements, we sought to determine whether SIK inhibition alters cytokine responses in disease-relevant tissues by
quantifying abundance of *Il10*, *Tnf* and *Il6* mRNA in the colon of mice treated as above. As observed for serum cytokine levels, YKL-05-099 pre-treatment significantly enhanced the LPS-induced increase in *Il10* mRNA at 1 hr, while *Tnf* transcript was reduced throughout the experiment (Figure 4.3D). Again, the LPS-induced increase in *Il6* mRNA was not affected by YKL-05-099 (Figure 4.3D). These data are consistent with the cell-based activity of YKL-05-099, and further support SIKs as a target to modulate systemic and tissue-specific inflammatory responses.

As a first step to determining whether acutely inhibiting SIKs with small molecules yields similar metabolic phenotypes as chronic SIK inhibition by genetic deletion, we treated mice with 20 mg/Kg YKL-05-099 daily for 1 week and we observed no changes in weight (Figure 4.5A). At the end of the treatment course, we measured fasting levels of serum triglycerides as well as high- and low-molecular-weight adiponectin. In contrast to *Sik2*−/− animals, daily dosing with YKL-05-099 did not alter serum levels of these adipocyte-derived hormones and metabolites (Figure 4.5).

Supplementary Figure 4.5: Extended treatment with YKL-05-099 does not induce metabolic abnormalities observed in *Sik2*−/− mice. A) Mice maintain weight when receiving once daily IP administration of 20 mg/Kg YKL-05-099 (mean ± SD, n = 4 mice, data is from 1 independent experiment). B) and C) Levels of total and high-molecular-weight (HMW) adiponectin or triglycerides were determined in mice treated with vehicle or YKL-05-099 (20 mg/Kg, IP) once daily for 7 days (bars = mean, n = 3 or 4 mice, data is from 1 independent experiment).

In this report, we describe optimization of the SIK-targeting inhibitor HG-9-91-01 to generate analogs suitable for use in animals. Docking HG-9-91-01 into the protein
kinase domain of the closely related AMPK-family member MARK3/Par-1 enabled these efforts by: 1) suggesting that HG-9-91-01’s core could be substituted with a fused pyrimidopyrimidinone in its bound conformation, and 2) identifying the ortho position on the aniline tail as a solvent-exposed site that could be substituted to improve SIK kinase selectivity. Applying these insights yielded YKL-05-099, a pan-SIK inhibitor that displays activity consistent with SIK inhibition (i.e., reduced SIK-specific phosphorylation of HDAC5) in cells and following direct injection into animals. As such, YKL-05-099 and related compounds may represent useful probes for study of SIK function in vivo.

In culture, SIK inhibition converts activated innate immune cells to an anti-inflammatory phenotype marked by up-regulation of IL-10 and reduced production of several inflammatory cytokines including TNFα\textsuperscript{17, 128}. Our data in mice pre-treated with YKL-05-099 prior to LPS stimulation indicates that SIK inhibition similarly affects inflammatory responses in vivo. In particular, the ability of YKL-05-099 to potentiate IL-10 and suppress TNFα in the colon argues for exploration of SIK inhibitors for treatment of inflammatory disorders affecting the gastrointestinal track such as Crohn’s disease\textsuperscript{134}. However, because aberrant production of IL-10 and TNFα are hallmarks of other autoimmune and auto-inflammatory disorders such as rheumatoid arthritis, psoriasis and psoriatic arthritis, SIK inhibition may be a broadly applicable therapeutic strategy\textsuperscript{135-137}.

A potential liability for therapeutic development of SIK inhibitors is the role of these kinases in glucose and fat metabolism as well as regulation of microtubule function\textsuperscript{118, 119, 138}. However, repeated, daily injection of YKL-05-099 in wild-type mice was well tolerated and did not recapitulate the effects of Sik2 deletion on serum triglyceride and adiponectin levels. At the 20 mg/Kg dose, free concentrations of YKL-05-099 are predicted to exceed
the IC\textsubscript{50} for SIK inhibition for > 16 hours, such that SIK activity may adequately recover between doses to normalize lipid handling in adipocytes. Nevertheless, this data suggests the presence of a therapeutic window that would allow for repeated dosing of SIK inhibitors to achieve the desirable immunomodulatory effects while preserving normal metabolic function.

4.1.3. Materials and Methods

In vivo methods. All animal studies were conducted under protocols approved by the Subcommittee on Research Animal Care at Massachusetts General Hospital. YKL-05-099 was diluted in 5% N-methyl-2-pyrrolidinone, 5% Solutol HS15 and 90% normal saline and administered IP to male 8 – 10 week-old C57BL/6 mice. Serum and tissue samples were collected after euthanizing mice by CO\textsubscript{2} inhalation overdose followed by cervical dislocation.

Cell culture and biochemical assays. Bone marrow-derived DCs or macrophages were differentiated from C57BL/6 bone marrow in the presence of GM-CSF-conditioned media or recombinant M-CSF, respectively. Inhibition of purified, recombinant hSIK2 was measured using a Caliper-based kinase activity assay (PerkinElmer). Concentrations of IL-10, TNF-\(\alpha\), IL-6, IL-12p40 and IL-1\(\beta\) in culture media or serum were detected using a FlexSet Cytometric Bead Array assay (BD Biosciences). Alternatively, IL-10 secretion was quantified using an AlphaLISA assay (PerkinElmer). qPCR was conducted using iTaq Universal SYBR Green Supermix (Bio-Rad) on total RNA prepared from cultured cells or distal colon sections. Details on reagents, chemical synthesis and full experimental protocols are available in the Supplementary Information.
AWKNOLEDGEMENTS

We thank the Broad Compound Management and Analytical Chemistry Groups for providing compound plates and conducting in vitro PK studies, respectively. We thank Kara Conway-Hoffman and Nicole Desch (Massachusetts General Hospital) for expert assistance with design of the animal studies. We thank the Nancy Lurie Marks Medicinal Chemistry Core for assistance with compound preparation.

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4.2. Small-molecule studies identify CDK8 as a regulator of IL-10 in activated myeloid cells (Manuscript under review at NCB)

Liv Johannessen1,2,13, Thomas B. Sundberg3,13, Daniel J. O’Connell13, Raivo Kolde5, James Berstler5, Katelyn J. Billings6,7, Bernard Khor5, Brinton Seashore-Ludlow7, Anne Fassl2,8, Caitlin N. Russell9, Isabel J. Latorre4, Baishan Jiang1,2, Daniel B. Graham4,10, Jose R. Perez3, Piotr Sicinski2,8, Andrew J. Phillips3, Stuart L. Schreiber7,11,12, Nathanael S. Gray1,2,*), Alykhan F. Shamji1,2, Ramnik J. Xavier4,5,9,*

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Below is a manuscript, currently under review at Nature Chemical Biology describing small molecule screening effort which identified a novel role for CDK8 in regulation of innate immune activation. Similar to the SIK inhibitors, CDK8 inhibition increases the transcription of IL-10, though through a previously unappreciated mechanism. I worked to confirm that this IL-10 phenotype was due to CDK8 inhibition and to elucidate the mechanism through which it occurred, identifying a new substrate for CDK8 (Figures 4.6-4.19). Additionally, I was wrote, made figures, and helped prepare the manuscript for submission. The paper is as follows:
4.2.1. Abstract

Enhancing production of the anti-inflammatory cytokine IL-10 is a promising strategy to suppress pathogenic inflammation. To identify new mechanisms regulating IL-10 production, we conducted an unbiased phenotypic screen for small molecules that enhance IL-10 production in activated dendritic cells. Mechanism-of-action studies with a prioritized hit from the screen (BRD6989) identified the Mediator-associated kinase CDK8, and its paralog CDK19, as negative regulators of IL-10 production during innate immune activation. The ability of BRD6989 to upregulate IL-10 is recapitulated by multiple, structurally differentiated CDK8/19 inhibitors and requires an intact CDK8/Cyclin C complex. Using a highly parallel pathway-reporter assay, we identified a role for enhanced AP1 transcriptional activity in IL-10 potentiation following CDK8/19 inhibition, an effect associated with reduced phosphorylation of a negative regulatory site on c-Jun. These findings identify a role for CDK8/19 in regulating innate immune activation and suggest that these kinases may warrant consideration as therapeutic targets for inflammatory disorders.

GRAPHICAL ABSTRACT
4.2.2. Innate Immunity and Dendritic Cells

The anti-inflammatory cytokine interleukin-10 (IL-10) promotes immune homeostasis by suppressing inflammatory cytokine production by innate immune cells and by promoting regulatory T cell (T\(_{\text{reg}}\)) function\(^{139}\). Genetic studies of inflammatory bowel disease (IBD) have linked single nucleotide polymorphisms near \(IL10\) to adult-onset IBD and identified rare, loss-of-function mutations in \(IL10\) or its receptor that result in severe, pediatric-onset enterocolitis\(^{134}\). Conversely, IL-10-based therapy reduces disease activity in murine models of colitis, bacterial infection, psoriasis and arthritis\(^{140}\). In addition, oral administration of bacteria engineered to express IL-10 or injection of a fusion protein that links recombinant IL-10 (rIL-10) to an inflammation-targeting antibody has shown promise in clinical trials for Crohn’s disease and rheumatoid arthritis, respectively\(^{141}, 142\). Hence, while impaired IL-10/IL10R signaling can lead to inflammation, increasing IL-10 abundance may be a viable therapeutic approach to restore and maintain immune homeostasis.

4.2.3. Importance of IL-10 In IBD

Small-molecule probes have provided key insights into regulation of IL-10 in innate immune cells. Activators of CREB-dependent transcription such as G protein-coupled receptor agonists or inhibitors of phosphodiesterases (PDEs), salt-inducible kinases (SIKs), or glycogen synthase kinase-3\(\beta\) (GSK-3\(\beta\)) potentiate IL-10 production in macrophages and dendritic cells\(^{17}, 128, 143, 144\). Alternatively, amplification of mitogen-activated protein (MAP) kinase signaling by perturbation of microtubule dynamics or inhibition of cyclin-dependent kinase 5 (CDK5) can also up-regulate IL-10\(^{64}, 145\). Notably, these probes have identified therapeutically useful drug targets, exemplified by approval
of the PDE4 inhibitor apremilast for treatment of psoriatic arthritis as well as pre-clinical evaluation for treatment of IBD\textsuperscript{135, 146}. Based on these successes, we reasoned that unbiased phenotypic screening for small-molecule enhancers of IL-10 production would identify novel mechanisms of IL-10 regulation and, potentially, new targets for development of anti-inflammatory therapies.

4.3. Results:

4.3.1. Identification of small-molecule enhancers of IL-10 production by unbiased phenotypic screening

Towards the goal of identifying new mechanisms of IL-10 regulation, we screened 59,346 small molecules for the ability to enhance IL-10 production by activated bone marrow-derived dendritic cells (BMDCs) using a high-throughput assay that we developed previously\textsuperscript{17}. Compounds were derived from both commercial collections and structurally and skeletally diverse libraries prepared by diversity-oriented synthesis (DOS). We identified >60 hit compounds that reproducibly enhance IL-10 production in a concentration-dependent manner (Table 4.2). We prioritized three distinct chemotypes for follow-up based on potency and an initial analysis of structure-activity relationship (SAR) with respect to core scaffold, stereochemistry and side chains (Figure 4.6a). Among these, the DOS-derived hits BRD0330 (1) and BRD0326 (7) were deprioritized based on data showing that they stimulated production of the inflammatory cytokine IL-1β or suppressed microtubule polymerization, respectively (Figure 4.7 and Figure 4.8).
Figure 4.6: BRD6989 prioritized from small molecule IL-10 enhancers identified by unbiased phenotypic screening. (a) Chemical structure of IL-10 enhancers identified unbiased phenotypic screening and their rationale for deprioritization. (b) Chemical structure of BRD6989. (c,d) Effect of BRD6989 on cytokine production in BMDCs stimulated with Zymosan A (c) or R848 (d) (each point corresponds to mean ± S.D.; n=3 biological replicates from 1 independent experiment). (e) Effect of BRD6989 on IL-10 production in human DCs stimulated with R848 (each point corresponds to mean ± S.D.; n=3 biological replicates from 1 independent experiment using cells derived from 1 donor). All data is representative of at least 2 independent experiments.
Table 4.2: Screening strategy for identification of novel small molecule IL-10 enhancers.

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<td>Assay</td>
<td>Type of assay</td>
<td>Unbiased phenotypic screen for small molecules that up-regulate IL-10 secretion by zymosan A-stimulated BMDCs.</td>
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<td>Primary</td>
<td>Measurement</td>
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<td>Mouse IL-10 AlphaLISA detection reagent (Perkin Elmer; cat AL502); Zymosan A (Sigma; cat Z4250)</td>
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<tr>
<td>Library</td>
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<td>Library composition</td>
<td>~47,000 compounds from the Broad Institute’s diversity-oriented synthesis library, which is a collection of compounds designed to maximize structural and stereochemical diversity. ~13,000 compounds from commercial screening libraries collected as part of the Molecular Libraries Program.</td>
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<td>Source</td>
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<td>Broad internal library and various commercial sources.</td>
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<td>Screen</td>
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<td></td>
<td>Plate controls</td>
<td>Negative control = DMSO; Positive control = PGE2 at final concentration of 5 μM</td>
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<td></td>
<td>Reagent/ compound dispensing</td>
<td>BMDCs and zymosan A were dispersed in 384 well plates using a Multidrop Combi Reagent Dispenser (Thermal Scientific) and compounds were pin-transferred using a CyBi-Well Vario (CyBio).</td>
</tr>
<tr>
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<td>Detection</td>
<td>instrument and software</td>
<td>AlphaLISA signal intensity was read using an EnVision multimode plate reader (Perkin Elmer)</td>
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<td>Assay validation/QC</td>
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<td>Plate effects were corrected using the Run-based non-parametric algorithm in the Genedata Screener software suite (Genedata).</td>
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<td>Normalization</td>
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<td>Compound activity was normalized as the percent difference in the IL-10 AlphaLISA signaling in the PGE2-versus DMSO-treated wells on a per plate basis.</td>
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<td>Post-HTS</td>
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<tr>
<td>Hit criteria</td>
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<td>Hits were classified as compounds that increased IL-10 production by an average of 30% of the PGE2 response from two replicates.</td>
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<td>Hit rate</td>
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<td>0.55% (331/59346)</td>
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<td>Additional</td>
<td>assay(s)</td>
<td>The ability of hit compounds to enhance IL-10 was confirmed by orthogonal detection strategies including ELISA and cytokine-bead array assays.</td>
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<tr>
<td>Confirmation</td>
<td>of hit purity and structure</td>
<td>Compound purity was determined by LC-MS of DMSO stocks used to confirm concentration-dependent enhancement of IL-10 production. BRD6989 structure and purity was further validated by purchasing powder from a commercial vendor.</td>
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<tr>
<td>Additional</td>
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Figure 4.7: DOS-derived IL-10 hit BRD0330 was deprioritized due to coordinated upregulation of IL-1β production. (a) Chemical structure of BRD0330. (b) BRD0330 upregulates IL-10 production by Zymosan A-stimulated BMDCs to a similar extent at PGE2 (each point correspond to mean ± S.D.; n=3 biological replicates from 1 independent experiment; data is representative of >4 independent experiments). (c) BRD0330’s IL-10 enhancing activity depends on its stereochemistry (EC \(_{50}\)’s = mean of 2 independent experiments). (d) Substitution of the stilbene side chain (R\(_1\)) increases potency the potency of IL-10 and IL-1β up-regulation by BRD-03330 in activated BMDCs (EC \(_{50}\)’s are determined from 1 independent experiment). (e) The potency of IL-10 and IL-1β enhancement correlates (R\(^2\) = 0.97) for BRD0330 and R\(_1\) analogs 2 – 6.
Figure 4.8: DOS-derived IL-10 hit BRD0326 perturbs microtubule dynamics. (a) Chemical structure of BRD0326. (b) BRD0326 potentiates IL-10 production by Zymosan A-stimulated BMDCs to a similar degree as PGE2 (each point corresponds to mean ± S.D.; n = 3 biological replicates from 1 independent experiment; data is representative of 2 independent experiments). (c) BRD0326’s IL-10 enhancing activity depends on its stereochemistry (EC50’s = mean of 2 independent experiments). (d) Transcriptional responses elicited by BRD0326 correlate with those induced by small molecule modulators of microtubule dynamics. (e) BRD0326, but not the IL-10 inactive stereoisomers BRD8765 or BRD6763, suppresses tubulin polymerization in a cell-free assay (data is from 1 independent experiment).
4.3.2. BRD6989 selectively upregulates IL-10 by a distinct mechanism

The pyridinyl tetrahydroquinoline BRD6989 (10) emerged as a prioritized hit in our screen (Figure 4.6a and Table 4.3). Pre-treatment of BMDCs with BRD6989 for 48 hr prior to stimulation with the yeast cell wall extract Zymosan A for 18 hr increased IL-10 production with an EC$_{50}$ ~1 μM (Figure 4.6b), while only modestly reducing cell viability in these assay conditions (Figure 4.9a). In addition, BRD6989 suppressed Zymosan A-induced release of the inflammatory cytokine IL-6, while leaving production of TNFα, IL-12p40 and IL-1β largely unchanged (Figure 4.6b and Figure 4.9b). BRD6989 induced similar cytokine responses in BMDCs stimulated with the viral RNA mimetic R848, but with a greater fold-increase in IL-10 production (Figure 4.6c and Figure 4.9b). Along with BMDCs, BRD6989 increased IL-10 production in mouse bone marrow-derived macrophages (BMDMs) activated with Zymosan A or R848 (Figure 4.9c). Lastly, BRD6989 up-regulated IL-10 following R848 stimulation in human, monocyte-derived DCs from two independent donors at concentrations consistent with its activity in BMDCs (Figure 4.6d. and Figure 4.9d). Thus, BRD6989 enhances IL-10 production in activated human and murine macrophages and dendritic cells by a mechanism that appears most pronounced following stimulation of toll-like receptors-7 (TLR7) and TLR8 by R848.

Analyzing the effects of BRD6989 pre-treatment on Il10 mRNA levels following R848 stimulation revealed a sustained increase beginning at 4 hours and extending to 24 hours (Figure 4.9f). These transcript dynamics contrast with activators of CREB-dependent transcription like the pan-SIK inhibitor HG-9-91-01 (11)$^{128}$, which elevate Il10 mRNA immediately following microbial stimulation without greatly extending the duration of this response (Figure 4.9f). Based on its ability to selectively potentiate IL-10
production by a mechanism that appears temporally distinct from CREB activation, we prioritized BRD6989 for mechanism-of-action (MoA) studies.

**Supplementary Table 4.3: Chemical Probe Data for BRD6989**

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<td></td>
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<tr>
<td><strong>In vitro profiling</strong></td>
<td>Target</td>
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<td>Potency</td>
<td>Cyclin C/CDK IC$<em>{50}$ ~0.5 μM and Cyclin C/CDK19 IC$</em>{50}$ &gt; 30 μM</td>
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<tr>
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<td>Selectivity</td>
<td>Profiling for binding or inhibition of 414 recombinant kinases identified Cyclin C/CDK8 as the primary kinase target of BRD6989</td>
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<tr>
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<td>Potential reactivities</td>
<td>None noted</td>
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<td>SAR</td>
<td>Pyridine and amino substituents as well as the methyl cyclohexyl core are required for CDK8 binding (See Supplementary Fig. 5)</td>
</tr>
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<td>Mechanism of inhibition</td>
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<td>Structure of target-probe complex</td>
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<td>Additional comments</td>
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<td><strong>Cellular profiling</strong></td>
<td>Validation of cellular target</td>
<td>Dose dependent inhibition of CDK8 dependent phosphorylation of STAT1 S727 phosphorylation.</td>
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<td>Validation of cellular specificity</td>
<td>(1) Multiple structural distinct CDK8 inhibitors phenocopy the IL-10 enhancing activity of BRD6989. (2) Genetic deletion of Cyclin C abrogates the IL-10 enhancing activity of BRD6989.</td>
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<td>Additional comments</td>
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Figure 4.9: Cellular activities of the IL-10 hit BRD6989. (a) BRD6989 does not significantly reduce the viability of zymosan A-stimulated BMDCs (each point corresponds to mean ± S.D.; n = 3 biological replicates from 1 independent experiment). (b) Pre-treatment of BMDCs with BRD6989 minimally elevates IL-1β production induced by stimulation with R848 or Zymosan A (each point corresponds to mean ± S.D.; n = 3 biological replicates from 1 independent experiment). (c) Pre-treatment of BMDMs with BRD6989 or DCA up-regulates IL-10 production by R848- or Zymosan A-stimulated BMDMs (each bar corresponds to mean ± S.D.; n = 3 biological replicates for 1 independent experiment). ***, P < 0.001 using one-way ANOVA with Dunnett’s post-test. (d) Effect of BRD6989 on IL-10 production in human DCs stimulated with R848 (each point corresponds to mean ± S.D.; n=3 biological replicates from 1 independent experiment using cells derived from 1 donor). (e) Chemical structure of the pan-SIK inhibitor HG-9-91-01. (f) Effect of pre-treating BMDCs with HG-9-91-01 (0.5 μM) or BRD6989 (5 μM) on Il10 mRNA levels following stimulation with R848 (each point corresponds to mean ± S.D.; n = 4 biological replicates from 1 independent experiment; *, P < 0.05; ***, P < 0.001 using unpaired, two-tailed Student’s t test). Data from all panels is representative of at least 2 independent experiments.
4.3.3. The Mediator-associated kinase CDK8 is a molecular target of BRD6989

Given that the pyridine core of BRD6989 is a common kinase-binding motif\textsuperscript{147}, we profiled its activity against 414 kinases using a series of binding and activity assays. We found that BRD6989 binds a complex of CDK8/Cyclin C, a Mediator-associated kinase not previously linked to IL-10 regulation\textsuperscript{4}, with an IC\textsubscript{50} ~200 nM and remarkable selectivity (Figure 4.10a, Figure 4.11a). Binding of BRD6989 to CDK8, and its paralog CDK19, was confirmed using an orthogonal kinase-profiling format. In addition to CDK8, both kinase profiling experiments identified phosphatidylinositol-4,5-biphosphate 3-kinases C2A (PI3KC2A) and PI3KCG as secondary targets of BRD6989 suggesting an overall consistency between the approaches. Of note, PI3K inhibitors with varying isoform specificity screened in our assay system fail to enhance IL-10 production\textsuperscript{17}, suggesting that inhibition of PI3Ks does not contribute to IL-10 potentiation by BRD6989.

In agreement with the kinase profiling results, BRD6989 inhibits the kinase activity of recombinant CDK8/Cyclin C or CDK19/Cyclin C complexes with IC\textsubscript{50}’s ~0.5 μM and >30 μM, respectively (Figure 4.10b), but not the activity of several CDKs involved in cell cycle regulation (Figure 4.11b-f). Furthermore, pre-incubation of BMDCs with BRD6989 inhibited IFN\gamma-induced phosphorylation of STAT1 at the known CDK8-regulated position S727\textsuperscript{148}, but did not affect JAK-mediated phosphorylation of Y701 (Figure 4.10c). Preliminary SAR analysis of BRD6989’s effects on CDK8 and IL-10 suggest that the pyridine and amino substituents and the methyl cyclohexyl core make critical contacts with CDK8 (Figure 4.12). Notably, the potency of CDK8 binding correlates with that of IL-10 induction for these analogs (Figure 4.10d), whereas BRD6989 only inhibits CDK19 at concentrations much greater than the EC\textsubscript{50} for IL-10 induction. Together, these kinase
profiling, binding assay, enzyme inhibition and cell-based data identify the Mediator-associated kinases CDK8, and to a lesser degree CDK19, as molecular targets of BRD6989.

Figure 4.10: CDK8 is a molecular target of BRD6989. (a) Kinase profiling identifies Cyclin C/CDK8 as a molecular target of BRD6989 (each point corresponds to the mean of 3 biological replicates from 1 independent experiment). (b) Effect of the indicated concentrations of BRD6989 on the activity of Cyclin C/CDK8 or Cyclin C/CDK19 complexes (each point corresponds to mean ± S.D.; n=3 biological replicates from 1 independent experiment for Cyclin C/CDK8 or n=2 biological replicates from 1 independent experiment for Cyclin C/CDK19). (c) BRD6989 suppresses phosphorylation of the STAT1 transactivation domain at S727 in IFNγ-stimulated BMDCs (data is representative of 3 independent experiments). (d) CDK8 binding affinity (IC₅₀) correlates with the potency of IL-10 up-regulation (EC₅₀) for BRD6989 and its derivatives (R² = 0.91) (IC₅₀ and EC₅₀ data is from 1 independent experiment).
Figure 4.11: BRD6989 binds to CDK8, and does not inhibit several other CDKs. (a) Binding of the indicated concentrations of BRD6989 to CDK8/Cyclin C complexes (each point corresponds to mean ± S.D.; n = 3 biological replicates from 1 independent experiment). (b-f) Effect of the indicated concentrations of BRD6989 on the activity of (b) Cyclin B/CDK1, (c) Cyclin A/CDK2, (d) p35/CDK5, (e) Cyclin H/CDK7, and (f) Cyclin T/CDK9 (each point corresponds to mean ± S.D.; n = 3 biological replicates from 1 independent experiment).
Figure 4.12: The potency of CDK8 binding and IL-10 potentiation by BRD6989 and related analogs are correlated. CDK8 binding (IC$_{50}$) as well as effects on IL-10 production (EC$_{50}$) by R848-stimulated BMDCs are from 1 independent experiment.

### 4.3.4. Pharmacological and genetic evidence supports CDK8 as a regulator of IL-10 production during innate immune activation

Burgeoning interest in Mediator-associated kinases as chemotherapeutic targets has prompted development of several structurally distinct CDK8/19 inhibitors including $\Delta^{16}$-cortistatin A (DCA; 17), an analog of the potent, CDK8/19-selective natural product cortistatin A$^{149, 150}$, and the recently published highly selective CDK8/19 inhibitor CCT251921 (18)$^{151}$ (Figure 4.13a). Pre-treatment with these CDK8/19 inhibitors
recapitulated the IL-10 potentiating activity of BRD6989 in both BMDCs (Figure 4.13b) along with human DCs derived from two independent donors in the context of R848 stimulation (Figure 4.13c and Figure 4.14a). Significantly, the IL-10 potentiating activity of these three inhibitors occurs as concentrations consistent with their potencies for CDK8/19 inhibition. In contrast, IL-10 production is not enhanced by the CDK7-targeting inhibitor THZ-1\textsuperscript{38} (Figure 4.14b), suggesting that this effect does not occur in response to inhibition of other transcriptional regulatory kinases. Lastly, the effect of CDK8 inhibition on IL-10 production may be cell type-specific because neither DCA nor BRD6989 dramatically increased IL-10 production during ex vivo differentiation of T\textsubscript{reg}'s (Figure 4.14c). Taken together, these data support CDK8 inhibition as the mechanism driving IL-10 potentiation in response to BRD6989 in activated human and murine DCs.

Next, to determine whether IL-10 potentiation by CDK8 inhibitors requires functional CDK8/Cyclin C complexes, we differentiated BMDCs from Ccnc\textsuperscript{Δ/Δ} mice in which Cyclin C can be specifically disrupted in hematopoietic cells relative to Ccnc\textsuperscript{Δ/Δ} littermates, which lack the polyinosolic:polycytidylic acid (polyI:C)-responsive Mx1-Cre\textsuperscript{94} (Figure 4.15a). Notably, there was not a significant difference in the levels of IL-10 secreted between Ccnc\textsuperscript{Δ/Δ} and Ccnc\textsuperscript{Δ/Δ} following R848 stimulation (Figure 4.15b), suggesting that other regulatory mechanisms can compensate for a sustained loss of CDK8 activity and maintain low basal levels of IL-10 production. However, Ccnc\textsuperscript{Δ/Δ} BMDCs failed to upregulate IL-10 in response to DCA or BRD6989, whereas the response remained intact in Ccnc\textsuperscript{Δ/Δ} BMDCs (Figure 4.13d, e and Figure 4.15 c,d). In addition, Ccnc\textsuperscript{Δ/Δ} BMDCs displayed a ~50% reduction in IL-6 production relative to Ccnc\textsuperscript{Δ/Δ} BMDCs and saturating concentrations of DCA or BRD6989 caused no further reduction
Cyclin C deletion does not appear to inhibit cytokine production non-specifically since it does not affect IL-10 and IL-6 responses induced by pan-SIK inhibition with HG-9-91-01 (Figure 4.15e). The impaired response of BMDCs lacking Cyclin C to BRD6989 and DCA are consistent with a model in which CDK8 restrains IL-10 production during activation of wild type myeloid cells.

Figure 4.13: Pharmacological and genetic data identifying CDK8 as a negative regulator of IL-10 production. (a) Chemical structures of the potent, specific CDK8/19 inhibitors CCT251921 and Δ16-cortistatin A (DCA). (b) CCT251921 and DCA recapitulate the IL-10 enhancing activity of BRD6989 in R848-stimulated BMDCs (each point corresponds to mean ± S.D.; n=3 biological replicates from 1 independent experiment). (b) CCT251921 and DCA recapitulate IL-10 potentiation by BRD6989 in human DCs stimulated with R848 (each point corresponds to mean ± S.D.; n=3 biological replicates from 1 independent experiment using cells derived from 1 donor). (d,e) Deletion of Cyclin C in BMDCs derived from CcnCΔ/Δ mice impairs induction of IL-10 and suppression of IL-6 by (d) DCA or (e) BRD6989 following activation with R848 (each point correspond to mean ± S.D.; n=4 biological replicates from 1 independent pair of CcnCΔ/Δ or CcnCfl/fl mice). All data is representative of at least 2 independent experiments.
Figure 4.14: Cellular activities of CDK8 inhibitors. (a) CCT251921 and DCA recapitulate IL-10 potentiation by BRD-6989 in human DCs stimulated with R848 (each point corresponds to mean ± S.D.; n=3 biological replicates from 1 independent experiment using cells derived from 1 donor). (b) Effect of CDK7 inhibitor THZ1 on viability and IL-10 production of R848-stimulated BMDCs (each point corresponds to mean ± S.D.; n = 4 biological replicates from 1 independent experiment). (c) IL-10 levels were quantified in tissue culture media generated during differentiation of naïve, splenic CD4+ T cells into T<sub>reg</sub>'s in the presence of DCA (0.1 μM) or BRD6989 (5 μM) (each point correspond to mean ± S.D.; n = 3 biological replicates from 1 independent experiment; *, P < 0.05; n.s., not significant using one-way ANOVA with Dunnett’s post-test). Data from all panels is representative of at least 2 independent experiments.
Figure 4.15. Upregulation of IL-10 by CDK8 inhibitors requires an intact CDK8/cyclin C complex. (a) Genotyping PCR of genomic DNA and immunoblotting of whole cell lysates confirms recombination of the Ccnc locus and reduction of Cyclin C levels, respectively, in BMDCs derived from CcncΔΔ mice. (b) Reduction of cyclin C levels in BMDCs does not alter basal levels of IL-10 production in response to R848 (each bar represents mean ± S.D.; n = 3 independent biological replicates from 3 pairs of CcncΔΔ versus Ccncff mice). (c,d) Effect of CDK8 inhibition by DCA or BRD6989 on IL-10 production by R848-stimulated BMDCs derived from CcncΔΔ or Ccncff mice (each point corresponds to mean ± S.D.; n = 3 biological replicates for 2 independent pairs of CcncΔΔ versus Ccncff mice). (e) Effect of pan-SIK inhibition by HG-9-91-01 (0.5 μM) on production of IL-10 and IL-6 by R848-stimulated BMDCs derived from CcncΔΔ or Ccncff mice (each point corresponds to mean ± S.D.; n = 3 biological replicates for 1 independent pair of CcncΔΔ versus Ccncff mice). ***, P < 0.001 using unpaired, two-tailed Student’s t test.
4.3.5. Pharmacological inhibition of CDK8 induces gene-specific transcriptional responses in activated myeloid cells

To define systematically the role of CDK8 during innate immune activation, we profiled the transcriptional responses elicited by DCA or BRD6989 following stimulation of BMDMs with a panel of 10 microbial ligands or Sendai virus for time points from 15 min to 4 hours. BMDMs were chosen for these experiments because, like human and mouse DCs, they upregulate IL-10 in response to CDK8 inhibition (Figure 4.9c), and they have been previously studied using the multiplexed pathway reporter assay described below\(^\text{152}\). In this experiment, the transcriptional responses elicited by BRD6989 and DCA were highly correlated (Figure 4.16a), again supporting CDK8 as a principle cellular target of BRD6989. For instance, both CDK8 inhibitors reduced expression of the interferon-inducible genes \textit{Ifit2}, \textit{Cxcl10} and \textit{Rsad2} (Figure 4.17b). Given that CDK8-dependent phosphorylation of STAT1 at Ser727 is required for expression of many IFN\( \gamma \)-inducible genes\(^\text{148}\), these suppressive effects suggest that CDK8 inhibition may limit autocrine interferon signaling following microbial stimulation.

In addition to suppressing interferon-inducible genes, BRD6989 and DCA increased abundance of a similar, small subset of transcripts in both quiescent and activated BMDMs (Figure 4.16b and Figure 4.17a). Consistent with the effects of CDK8 inhibition on cytokine responses in activated myeloid cells, the induced genes include chemokines, metallopeptidases and regulators of cellular second messengers linked to inflammatory responses and migration of innate immune cells \((P = 0.0255\) and \(P = 1.52 \times 10^{-5}\), respectively). In the context of acute myeloid leukemia (AML), CDK8 inhibition preferentially induces genes near ‘super enhancers’ (SEs) defined by dense binding of the Mediator complex and transcription factors along with accumulation of histone
modifications associated with active transcription\textsuperscript{150}. Despite the limitations in comparing datasets from different myeloid cell types, we queried genes upregulated in BRD6989- or DCA-treated BMDMs for the presence histone modifications linked to active transcription previously identified in lipopolysaccharide (LPS)-stimulated BMDCs\textsuperscript{153}. In this analysis, mono-methylated Lys-4 on histone 3 (H3K4me1), a mark associated with poised enhancers\textsuperscript{154, 155}, is enriched ($P = 0.0093$) near genes induced by CDK8 inhibition relative to all highly expressed genes (Figure 4.16c). Although we detect a similar trend towards enrichment of histone 3 Lys27 acetylation (H3K27ac), a modification associated with active enhancers\textsuperscript{154, 155}, near genes induced by CDK8 inhibition, this association did not achieve statistical significance (Figure 4.16c). These data indicate that CDK8 primarily represses a small set of genes in quiescent or activated myeloid cells, although the linked between CDK8-regulated genes and histone modifications associated with active transcription appears less well correlated in this context.
Figure 4.16: Modulation of c-Jun/AP-1 links CDK8 inhibition to enhanced IL-10 production. (a) CDK8 responsive signaling pathways in activated BMDMs as identified by the highly parallel reporter gene assay TF-Seq. Reporter data is integrated for time points between 15 min – 4 hr from 1 independent experiment (See Online Methods). Dashed lines correspond to FDR <0.05 as corrected by Benjamini–Hochberg testing. (b) Relative to all highly expressed genes, JunB binding sites are enriched among the subset of genes induced by CDK8 inhibition (see Online Methods). (c) Pre-treatment with DCA (100 nM; 18 hr) suppresses phosphorylation of c-Jun on Ser243 in BMDCs activated with R848 (2 μg/mL) for the indicated times. Data is representative of 2 independent experiments. (d) The indicated concentrations of DCA suppress phosphorylation of recombinant c-Jun at Ser243 by recombinant Cyclin C/CDK8 in an in vitro kinase reaction. Data is representative of 2 independent experiments. (e) Co-treatment of BMDCs with the c-Fos/AP1 inhibitor T-5224 (100 μM) suppresses the IL-10 enhancing activity of DCA in BMDCs stimulated with R848 or Zymosan A (each point correspond to mean ± S.D.; n = 3 biological replicates from 1 independent experiment; data is representative of 3 independent experiments). CpG, synthetic CpG-containing oligonucleotide; FLA, flagellin A; LPS, lipopolysaccharide; MDP, muramyldipeptide; Pam3cys, synthetic triacylated lipoprotein; poly(I:C), polyinosinic-polycytidylic acid; SeV, Sendai virus; TDB, trehalose-6,6-dibehenate.
Figure 4.17: CDK8 inhibition increases expression of small subset of genes in activated and quiescent macrophages. (a) Changes in gene expression induced by the CDK8 inhibitors DCA and BRD6989 in activated BMDMs are highly correlated. (b) Inhibition of CDK8 with the structurally distinct inhibitor DCA and BRD6989 up-regulates expression of a small subset of genes in both quiescent BMDMs and following stimulation with a variety of microbial products. For (a,b), gene expression data is integrated from time points from 15 min – 4 hr from 1 independent experiment (see Online Methods); dashed lines correspond to FDR < 0.05 as corrected by Benjamini–Hochberg testing. (c) Enrichment of mono-methylated Lys-4 on histone 3 (H3K4me1) and acetylated Lys-27 on histone 3 (H3K27ac) among the subset of genes induced by CDK8 inhibition relative to all highly expressed genes (see Online Methods). CpG, synthetic CpG-containing oligonucleotide; FLA, flagellin; LPS, lipopolysaccharide; MDP, muramyldipeptide; Pam3cys, synthetic triacylated lipoprotein; Poly(l:C), polyinosinic-polycytidylic acid; SeV, Sendai virus; TDB, trehalose-6,6-dibehenate.
4.3.6. CDK8 inhibition upregulates IL-10 by a mechanism that involves suppressing inhibitory phosphorylation of c-Jun and enhancing AP-1 activity

To identify CDK8-responsive signaling pathways, we applied an integrative genomics approach termed transcription factor sequencing (TF-Seq) in which consensus transcription factor binding sites drive expression of 58 reporter constructs bearing unique sequence tags\textsuperscript{152}. Consistent with the established role of CDK8 in STAT1 activation\textsuperscript{148}, both BRD6989 and DCA suppressed induction of STAT1:STAT2 activity ~2 hours after stimulation of BMDMs (Figure 4.16a, Figure 4.18a). Also similar to previous studies of CDK8 function in other contexts including RPMI8226 myeloma cells activated by TLR9 stimulation\textsuperscript{95, 105, 156}, pre-treatment with DCA or BRD6989 suppressed NF-κB activation to a varying degree for all stimuli tested (Figure 4.16a and Figure 4.18b). Because DCA pre-treatment does not perturb upstream signaling events linking sensing of LPS or R848 to NF-κB activation (Figure 4.18c,d), it appears that inhibiting CDK8 may suppress the ability of nuclear NF-κB to activate transcription.

TF-seq also indicates that CDK8 inhibition increases activator protein 1 (AP1) activity in BMDMs (Figure 4.16a and Figure 4.19a). In support of this observation, binding sites for the AP1 subunit JunB are enriched among genes up-regulated following CDK8 inhibition relative to all highly expressed genes (Figure 4.16b). Given that c-Jun’s ability to activate transcription is tightly regulated by phosphorylation, we asked whether CDK8 inhibition affects c-Jun phosphorylation in activated BMDCs. For instance, activating phosphorylation of c-Jun on Ser63, which is rapidly induced by R848 stimulation and peaks after 30 minutes, is moderately delayed by pre-treatment with DCA (Figure 4.16c). In contrast, inhibiting CDK8 with DCA dramatically reduced
phosphorylation of Ser243, a suppressive mark shown to destabilize c-Jun and interfere with its ability to bind DNA\textsuperscript{18, 157, 158}, which is first observed 60 minutes after R848 stimulation (Figure 4.16c). Significantly, the inhibitory effects of DCA on c-Jun Ser243 phosphorylation appears to be concentration-dependent in both R848-stimulated BMDCs (Figure 4.19b), and in an in vitro kinase reaction with recombinant CDK8/Cyclin C and c-Jun (Figure 4.16d). Together, these data identify c-Jun Ser243 as a direct CDK8 substrate that regulates AP-1 activity in activated myeloid cells.

Given the presence of an AP1 consensus motif in \textit{Il10} and evidence that potentiation of MAP kinase signaling can increase IL-10 production\textsuperscript{64, 139, 145}, we hypothesized that increased AP1 activity due to reduced c-Jun Ser243 phosphorylation may mediate activation of IL-10 following CDK8 inhibition. Supporting this, we found that co-treatment of BMDCs with DCA and T-5224 (19), a small-molecule inhibitor of AP1 reported to bind c-Fos and inhibit its dimerization with c-Jun\textsuperscript{159} (Figure 4.19c), suppressed IL-10 production following stimulation with R848 or Zymosan A (Figure 4.16e). Importantly, T-5224 co-treatment did not affect the increase in IL-10 production resulting from potentiation of CREB signaling by pan-SIK inhibition with HG-9-91-01, or suppress R848-induced inflammatory cytokine production (Figure 4.19d,e). These results suggest that CDK8 inhibition upregulates IL-10 production by a c-Jun/AP1-dependent mechanism that is distinct from cAMP/CREB signaling.
Figure 4.18: CDK8 inhibition suppresses STAT1 and NF-κB activity during innate immune activation. Time course of (a) STAT1 and (b) NF-κB reporter activity in BMDMs pre-treated with DCA (0.1 μM) or BRD6989 (5 μM) prior to stimulation with the indicated viruses or microbial ligands (each point corresponds to TF-Seq reporter data derived from 1 independent experiment). (c,d) Pre-treatment with DCA (0.1 μM) does not suppress upstream signaling events leading to NF-κB activation in BMDCs stimulated with (c) LPS (100 ng/mL) or (d) R848 (2 μg/mL) for the indicated time points. Immunoblotting data is representative to 2 independent experiments. CpG, synthetic CpG-containing oligonucleotide; FLA, flagellin; LPS, lipopolysaccharide; MDP, muramyl dipeptide; Pam3cys, synthetic triacylated lipoprotein; Poly(I:C), polyinosinic-polycytidylic acid; SeV, Sendai virus; TDB, trehalose-6,6-dibehenate.
Figure 4.19: CDK8 inhibition enhances AP-1 activity during innate immune activation. (a) Time course of AP-1 reporter activity in BMDMs pre-treated with DCA (0.1 μM) or BRD989 (5 μM) prior to stimulation with the indicated viruses or microbial ligands for the indicated time points (each point corresponds to TF-Seq reporter data derived from 1 independent experiment). (b) Pre-treatment of BMDCs with the indicated concentrations of DCA for 18 hr suppresses R848-induced phosphorylation of c-Jun on Ser243. Data is from 1 independent experiment. (c) Chemical structure of the c-Fos/AP1 inhibitor T-5224. (c) Co-incubation of BMDCs with T-5224 (100 μM) and the indicated concentration of the pan-SIK inhibitor HG-9-91-01 does not affect up-regulation of IL-10 or suppression of IL-6 in Zymosan A-stimulated BMDCs (each data point corresponds to mean ± S.D.; n = 3 biological replicates for 1 independent experiment). (d) Co-incubation of BMDCs with T-5224 (100 μM) and the indicated concentration of the DCA does not affect production of IL-6 or TNFα in R848-stimulated BMDCs (each data point corresponds to mean ± S.D.; n = 3 biological replicates for 1 independent experiment). Data in panels (c,d) are representative of 2 independent experiments. CpG, synthetic CpG-containing oligonucleotide; FLA, flagellin; LPS, lipopolysaccharide; MDP, muramyldipeptide; Pam3cys, synthetic triacylated lipoprotein; Poly(I:C), polyinosinic-polycytidylic acid; SeV, Sendai virus; TDB, trehalose-6,6-dibehenate.
4.4. Discussion

4.4.1. Small molecule screening in primary immune cells allows for identification of a novel target in innate immune activation
Using unbiased phenotypic screening and MoA studies, we have identified the Mediator-associated kinase CDK8, and likely CDK19, as regulators of IL-10 production, a mechanism that might be harnessed to enhance anti-inflammatory functions of innate immune cells. Our initial screen uncovered three new structural classes of small molecules that enhance IL-10 production, including a probe found to bind to CDK8 through kinase profiling (BRD6989). The growing interest in Mediator-associated kinases as therapeutic targets in cancer has spurred development of several chemically distinct CDK8/19 inhibitors such as the potent, dual CDK8/19 inhibitor CCT251921, which shares pyridinyl tetrahydroquinoline core with BRD6989, and a close analog of the natural product cortistatin A (DCA). Though not as potent as these inhibitors, BRD6989 appears unique in its ability to differentially inhibit CDK8 relative to its paralog CDK19, suggesting it may inform further development of CDK8-specific inhibitors. Importantly, we found that both CCT251921 and DCA, like BRD6989, promote IL-10 production in R848-stimulated DCs in a manner that depends on an intact Cyclin C/CDK8 complex. Together, these data identify CDK8 as a druggable regulator of IL-10 production in activated myeloid cells.

4.4.2. CDK8 promotes tolerance in innate immune system
Relative to CDK7 and CDK9, which broadly affect transcriptional initiation by activating phosphorylation of the C-terminal domain of RNAP polymerase II, CDK8’s function appears to be more context-dependent, having been linked to specific effects on
interferon, TGFβ, Wnt and Notch signaling\textsuperscript{4}. For example, phosphorylation of the transactivation domain of STAT1 by CDK8 is required for expression of many interferon-inducible genes\textsuperscript{148}, and inhibiting CDK8 in AML lines with cortistatin A specifically elevates expression of SE-associated genes\textsuperscript{150}. Similarly, our transcriptional profiling data in BRD6989- or DCA-treated BMDMs suggests that CDK8’s kinase function primarily restrains expression of a limited number of genes in myeloid cells. In contrast, our preliminary analysis suggests that genes regulated by CDK8 in myeloid cells are less correlated with regions of active transcription (i.e., SE-associated), although it will be of interest to address this question in more detail.

Measuring the response to CDK8 inhibition using a multiplex pathway reporter assay (TF-Seq) provided insights into CDK8-sensitive signaling pathways in activated myeloid cells. Consistent with previous studies, we observed reduced activation of STAT1:STAT2 and a corresponding decrease in expression of several interferon-inducible genes. We also observe a decrease in NF-κB activity to a varying degree with different microbial stimuli, but which does not appear sufficient in magnitude to suppress expression of NF-κB targets on a genome-wide scale. This may explain why CDK8 inhibition does not suppress expression of \textit{Il10}, itself an NF-κB target gene\textsuperscript{161}. Furthermore, the observation that the increase in \textit{Il10} transcript in BRD6989-treated BMDCs occurs after the rapid burst of NF-κB activity triggered by microbial stimulation is consistent with a model in which CDK8 regulates \textit{Il10} expression through an NF-κB-independent mechanism.–These observations contrast with a recent report that identifies an essential role CDK8/19 in NF-κB-dependent \textit{Il10} expression in a B cell-derived myeloma cell line responding to TLR9 stimulation\textsuperscript{156}. While differences in cell type and microbial stimuli may alter the degree to
which NF-κB activation depends on CDK8/19, contrasting effects of depleting CDK8/19 by RNA interference versus specifically inhibiting their kinase activity with small molecules, which should not disrupt their kinase-independent roles in the Mediator complex⁴, may also play a role.

4.4.3. CDK8 regulates IL-10 through a novel mechanism involving direct phosphorylation of AP-1

CDK8 inhibition also increased AP1 reporter activity in the TF-Seq assay for nearly all microbial stimuli tested, and, binding sites for the AP1 subunit JunB are enriched near genes upregulated in response to CDK8 inhibitors. Enhanced AP1 activity appears to be important for potentiation of IL-10 following CDK8 inhibition as the Fos-targeting inhibitor T-5224 suppresses this response. In contrast, disrupting AP1 activity does not impair the ability of BMDCs to increase IL-10 production through a parallel, CREB-dependent pathway triggered by the pan-SIK inhibitor HG-9-91-01. Reduced phosphorylation of the negative regulatory site Ser243 on c-Jun may be central to the mechanism linking CDK8 inhibition to enhanced AP1 transcriptional activity and IL-10 production in myeloid cells. Of note, CDK8 appears to directly phosphorylate Ser243 in a cell-free biochemical assay in a DCA-dependent manner. Our data identifying CDK8 as a negative regulator of c-Jun/AP1 in activated myeloid cells contrasts with the role for CDK8 in promoting AP1 activity during serum stimulation of prostate cancer cells¹⁶. As such, while CDK8 appears to regulate c-Jun Ser243 phosphorylation following microbial stimulation of post-mitotic BMDCs, other kinases targeting this site (e.g., casein kinase-2 and Dyrk2a¹⁵⁷,¹⁵⁸) may play this role in cycling cancer cells.
Our data identify the CDK8/c-Jun module as a regulator of IL-10 production that is distinct from previously described pathways like cAMP/CREB signaling. It remains to be determined whether using CDK8 inhibitors to upregulate IL-10 will be an efficacious and tolerated treatment for inflammatory disorders. The clinical observations that targeted delivery of IL-10 to inflamed tissues can suppress inflammation suggest that recapitulating this strategy with small molecules may hold promise\textsuperscript{141, 142}. Notably, CDK8 selectively upregulates IL-10 production by activated myeloid cells suggesting its immunomodulatory activity could be localized to sites of inflammation circumventing dose-limiting toxicities associated with systemic delivery of IL-10\textsuperscript{162, 163}. In addition, given that increased IL-10 expression and other mechanisms that suppress anti-tumor immune responses have well-established links to tumor progression and resistance to chemotherapy\textsuperscript{164-166}, the anti-inflammatory effects of CDK8/19 inhibition described here warrant consideration in the context of targeting these kinases in cancer.

4.4.4. CDK8 viability as a therapeutic target in cancer
Given that CDK8/19 are fundamental regulators of transcription in numerous cell types, it is not surprising that small-molecule inhibitors of these Mediator-associated kinases are poorly tolerated in vivo at doses that achieve the sustained target occupancy required for anti-tumor activity\textsuperscript{73}. Although these toxicities may preclude therapeutic targeting of CDK8/19, it is possible that the anti-inflammatory consequences of CDK8/19 inhibition can be achieved in vivo using less aggressive dosing regimens. Our data suggest that inhibiting CDK8/19 affects a limited number of pathways in myeloid cells including several targeted by clinically approved inhibitors of Janus kinases and the proteasome, respectively\textsuperscript{167, 168}. Thorough investigation of potential toxicities associated
with immunomodulatory doses of CDK8/19 inhibitors in vivo will be a critical next step for
evaluating the potential of developing therapeutics targeting these Mediator-associated
kinases for treatment of inflammatory disorders.

4.5. Materials and Methods
High-Throughput Detection IL-10 Up-Regulation and Cell Viability. BMDCs were
seeded into black 384-well plates (Corning) at 20,000 cells per well in 40 μL complete
DMEM using a Multidrop Combi reagent dispenser (Thermal Scientific) followed by
incubation at 37 °C for 2 h to allow for plate adherence. Compounds (100 nL per well)
were pin-transferred from concentrated DMSO stocks using CyBi-Well Vario (CyBio) into
duplicate plates. For each treatment plate, 32 out of 384 wells were pinned with DMSO
as negative control and 32 out of 384 wells were pinned with PGE2 (5-μM final
concentration). Two days after compound treatment, BMDCs were stimulated with
zymosan (4 μg/mL final concentration) dispersed in culture medium (20 μL per well) using
a Multidrop Combi reagent dispenser. After 18 h, 5 μL of resulting supernatant was
transferred from the culture plate to white 384-well AlphaLISA plates (Perkin-Elmer) using
a CyBi-Well Vario. IL-10 abundance in the supernatants was determined using an
AlphaLISA assay (PerkinElmer) according to the manufacturer’s protocol and signal
intensity measured using an EnVision multimode plate reader. Compound activity was
expressed as a percent of the differences between the mean abundance of IL-10 in PGE2
versus DMSO wells on a per-plate basis. Cell viability assays were conducted on the
same tissue culture plates as the IL-10 assay. First media was removed and replaced by
solution of 50% (vol/vol) CellTiter-Glo (Promega) in PBS using a Multidrop Combi reagent
dispenser. Luminescence was read using an Envision multimode plate reader, and signal intensity calculated relative to the mean of DMSO control wells on a per-plate basis.

**Reagents.** Compounds were plated by the Broad Institute Compound Management Group in 10-point twofold dilution series and confirmed to have ≥95% purity by HPLC-MS. Prostaglandin E2 (PGE2), Zymosan A from *S. cerevisiae* were purchased from Sigma. Ultrapure Lipopolysaccharide (LPS) from *E. coli* O111:B4, and R848 were purchased from InvivoGen.

**CDK8/CDK19 kinase activity assay.** IC$_{50}$ ’s for BRD6989 were measured using the commercial service ProQinase ™. In brief, BRD6989 treated in dose against purified recombinant CDK8/CyclinC and CDK19/CyclinC and then amount of ATP-gammaP32 phosphate transfer to their recombinant RBER-IRStide was measured via a radiometric filter binding assay.

**CDK8/Cyclin C binding assays.** The potency of BRD6989 analog binding to hCDK8/CyclinC were determined using Lanthascreen™ binding assays according to the manufacturer’s protocol (Life Technologies).

**Isolation and culture of innate immune cells.** Bone marrow was flushed from femurs and tibias of C57BL/6 mice as described previously. Briefly, bone marrow-derived dendritic cells (BMDCs) were differentiated in DMEM supplemented with 2 mM GlutaMAX, 10% (vol/vol) FBS, penicillin, streptomycin, and 2% (vol/vol) mouse granulocyte-macrophage colony-stimulating factor (GM-CSF)–conditioned media derived from murine L cells. Cultures were differentiated for 7 d and routinely analyzed for >90% CD11c (allophtocyanin (APC)-conjugated anti-CD11c clone HL3; BD Biosciences) positivity by flow cytometry before use in experiments. Bone marrow-derived
macrophages (BMDMs) were differentiated under analogous culture conditions except for the use of macrophage colony-stimulating factor (M-CSF; Peprotech) at final concentration to 50 ng/mL. Cultures were supplemented with additional M-CSF-containing media after 4 d and harvested for experiments after 7 d of total culture. BMDMs were routinely analyzed for >90% positivity for CD11b (phycoerythrin (PE)-conjugated anti-CD11b clone M1/70; BD Biosciences) and F4/80 (APC-conjugated anti-F4/80 clone BM8; BioLegend). Unless otherwise indicated, cells were treated for 24 hr with BRD6989 (5 μM), DCA (100 nM), HG-9-91-01 (1 μM) or an equivalent concentration of DMSO (≤0.5%) and then stimulated for 18 hr with R848 (2ug/mL), LPS (50 ng/mL), or Zymosan A (4 μg/mL).

**CCNC mice:** CCNC^fl/fl^ and CCNC^fl/fl^/Mx1Cre mice previously generated in the Sicinisky lab were breed out and confirmed via genotyping (Li, N et al., 2014). 6-8 week pups were induced using 3 injections of pl:C over 10 days. On day 10, mice were sacrificed and bones harvested for isolation of BMDCs as described.

**High throughput IL-10 detection and cytotoxicity.** Concentration-dependent effects of BRD6989 analogs on IL-10 release and cellular cytotoxicity in R848 (2ug/mL), LPS (50ng/mL), and Zymosan A(4ug/mL) stimulated BMDCs were measured using ELISA IL-10 detection (BD biosciences) or CellTiterGlo (Promega) as described previously. EC_{50} values for IL-10 up-regulation and cellular cytotoxicity (CC_{50}) values were calculated using nonlinear regression in Prism6 (GraphPad).

**Immunoblotting.** Cell lysis and immunoblotting were conducted as described previously. Briefly, single cell suspensions were rinsed in ice-cold PBS and extracted in lysis buffer [50 mM Tris·HCl at pH 7.4, 1 mM EDTA, 50 mM NaF, 10 mM sodium β-
glycerol 1-phosphate, 1 mM DTT, 1 mM sodium orthovanadate, 1% (vol/vol) Triton X-100, 0.2% (w/vol) SDS and 1 × Complete EDTA-free Protease Inhibitor Mixture (Roche)]. Cell extracts were clarified by centrifugation at 14,000 × g for 10 min at 4 °C, and protein concentrations were determined using the Bradford assay (Bio-Rad). To detect proteins in cell lysates, 10 μg of protein extract was separated by SDS/PAGE. After transfer to PVDF membranes, proteins were detected by immunoblotting and visualized by treating the blots with SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific) followed by autoradiography. The following antibodies were used for immunoblotting: β-actin (clone AC-74) was from Sigma Aldrich; Cyclin C was from BD Biosciences; CDK8 antibody was obtained from Bethyl; tSTAT1, pSTAT1 Y701, pSTAT1 S727, pERK1/2 T202/Y204; pP38 T180/Y182, CDK5, p35/25, tIKBa, plIKKα/β S176/180, tIKKα, tIKKβ, pNFκB p65 S536, t NFκB p65 were from Cell Signaling. Band intensities were quantified using ImageJ170.

mRNA isolation. mRNA was isolated from cultured cells using a Dynabeads mRNA DIRECT Kit (ThermoFisher Scientific) according to the manufacturer’s protocol. Briefly, culture media was removed from BMDMs cultured in a 96 w plate and cells washed with PBS before suspension in lysis buffer. Lysates were incubated with Oligo (dT)25 Dynabeads at rt for 5 min. Beads were magnetically separated from the lysate and washed 3 × with Wash Buffer A and 1 × with Wash Buffer B before transfer to clean 96 well plate. mRNA was released suspension of beads in dH2O and beads removed magnetically. cDNA was prepared from purified mRNA using an iScript cDNA Synthesis kit (Bio-Rad) and diluted 1/10 in dH2O.
**qPCR.** Diluted cDNA was quantified by qPCR using the iQ SYBR Green Supermix (Bio-Rad) on a CFX96 real-time system (Bio-Rad). The relative abundance of each mRNA was calculated from Ct values using the $2^{-\Delta\Delta\text{Ct}}$ method\(^{171}\) and normalized relative to the abundance of $B2m$ mRNA. The primers used are as follows:

$Iil10$-F, 5’-GCTCTTACTGACTGGCATGAG-3’
$Iil10$-R, 5’-CGCAGCTCTAGGAGCATGTG-3’
$Cdk5r1$-F, 5’-GCCCTTCCTGGTAGAGCTG-3’
$Cdk5r1$-R, 5’-GTGTGAAATAGTGTGGTCGG-3’
$B2m$-F, 5’-TTCTGGTGCTTGTCTCACTGA-3’
$B2m$-R, 5’-CAGTATGTTCGGTCCCATTC-3’

**Multiplexed cytokine quantification.** After stimulation of BMDCs, culture medium was removed and clarified by centrifugation for 5 min at 14,000 × g. Mouse serum was diluted 1/4 in PBS prior to quantification of cytokines. Levels of TNF-α, IL-6, IL-10, IL-12p40 or IL-1β in culture medium or diluted mouse serum were quantified using FlexSet Cytokine Bead Array (BD Biosciences) according to the manufacturer’s protocol.

**Kinase profiling.** BRD6989 (10 μM) was tested for their ability to inhibit the activity of 414 kinases at the Invitrogen™ kinase profiling\(^{23}\). Results are presented as percent remaining kinase activity relative to DMSO control.

**Statistical analysis.** Unless otherwise indicated, error bars represent mean ± SD for three replicates from an independent experiment that is representative of at least two independent experiments. $EC_{50}$’s for IL-10 up-regulation, $CC_{50}$’s for cellular cytotoxicity and $IC_{50}$’s for CDK8 inhibition were estimated using the SmartFit Nonlinear regression in
Genedata Screener software suite (Genedata). Statistical significance of differences between experimental groups was assessed using unpaired, two-tailed Student $t$ test or two-way ANOVA with Dunnett post-test as indicated. Statistical significant calculations were conducted using Prism6 (GraphPad). Unless otherwise indicated, $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

**Simultaneous signaling pathway activity inference and global gene expression analysis.** Bone marrow derived macrophages (BMDMs) were differentiated in pen/strep containing DMEM plus 30% FBS and 10% L929 M-CSF conditioned medium (CMG). BMDMs were transduced with an equimolar pool of lentiviral sequencing-based gene reporters (TF-seq) in 90% concentrated lentiviral supernatant + 10% CMG on day 4. On day 7 BMDMs were frozen in 90% FBS and 10% DMSO and stored in liquid nitrogen. BMDMs were thawed in DMEM plus 30% FBS and 10% L929 M-CSF for 2 days before they were scraped off of petri dishes (BD Falcon), counted on a hemocytometer, and plated overnight in DMEM +10% FBS at 100,000 cells/well in 96-well tissue-culture treated plates (Corning). BMDMs were pretreated with CDK8 small molecule inhibitors for 30 min at 37°C prior to stimulation with a panel of innate immune ligands in a short 3 hour time series. After defined periods of stimulation, cells were lysed in RLT lysis buffer (Qiagen) and stored at -80°C. Total RNA was precipitated in 10% PEG-8000 and 1.5 M NaCl and the washed once in 10% PEG-8000 and 1.5 M NaCl. Purified total RNA was then then resuspended in 50 μL with 2 units TURBO DNase (Life Technologies) and incubated at 37°C for 30 min. The DNase was denatured by adding 75 μL of RLT lysis buffer and the remaining RNA re-precipitated and washed twice in 10% PEG-8000 and 1.25 M NaCl. Maxima reverse transcriptase (Thermo Scientific) was run according to the
manufacturer’s instructions using a multiplexed primed reverse transcriptase reaction containing 96-well sequence tagged gene reporter primers, specific to luciferase, and 96-well tagged polydT primers were used at 750 nM and 250 nM final concentrations respectively with 50 units of Maxima. After sequencing-tagging all first-strand cDNA during reverse transcriptase, each 96-well plate was pooled and the unincorporated primers were washed away from the cDNA by precipitating with 10% PEG-8000 and 1.25 M NaCl. After RNase A treatment at 37°C for 30 minutes, 10% of the first strand cDNA was used in a PCR reaction with Illumina-compatible sequencing primers targeted to the luciferase gene reporter transcripts. The gene reporter amplicon was then sequenced using a 50 cycle PE Illumina Miseq kit. The remaining first-strand cDNA was then converted to double-strand cDNA using the Second Strand Synthesis Module (NEB). 1 ng of the double-strand cDNA (ds-cDNA) libraries was then tagmented with Nextera XT (Illumina) according to the manufacturer’s protocol. The pooled cDNA libraries for global gene expression are sequence-tagged only on the 3’ end of the sense transcript, therefore, after full-length ds-cDNA tagmentation we performed enrichment PCR of only the sequence-tagged 3’ end of the mRNA transcript by combining the Nextera N700 series of primers with the Tru-Seq P5 adapter by means of sequence complementarity introduced during reverse transcriptase priming. This process creates 3’ digital gene expression libraries and was sequenced using 1 flow cell on the Illumina NextSeq machine.
Chapter 5: CDK8 promotes tolerance in adaptive immunity
Abstract

Maintaining the balance between inducing the differentiation of naïve CD4+ T cells into pro-inflammatory Th1 and Th17 cells and anti-inflammatory T regulatory (Treg) cells is critical for immune homeostasis. Elucidation of the pathways that regulate CD4+ differentiation into either pro- or anti-inflammatory states may identify new areas for therapeutic intervention in inflammatory disease. Previous work in the Xavier lab utilized primary cell screening to identify small molecules and pathways that can drive tolerogenic programs in both innate and adaptive immune cells, (SIK inhibitors in DCs and DYR1KA inhibitors in T cells). Despite frequent conservation of related genetic programs, SIK and DYRK1A inhibitors exemplify a general observation that many of these inhibitors tend to selectively work to regulate either the innate or adaptive immune systems. We identified inhibition of CDK8 as having a pro-tolerogenic effect on dendritic cells during innate immune activation, promoting production of IL-10 through direct phosphorylation of AP1. As AP1 is a transcription factor known to have an important role in T cell differentiation, we endeavored to see if CDK8 inhibition could also have a role in adaptive immunity. Utilizing in vitro systems developed by Bernard Khor in the Xavier Lab (MGH) we determined that CDK8 inhibition promotes differentiation of specifically anti-inflammatory Tregs and not pro-inflammatory Th1, and Th17 cells. These cells are functional in both in vitro and in vivo systems. Thus, CDK8 inhibition may be a novel and interesting target in inflammation, promoting tolerogenic programs in both innate and adaptive immunity.
5.1. Introduction

5.1.1. Introduction to Adaptive immunity, inflammation, CD4+ T cells

Both the innate and the adaptive immune systems play separate but important roles in maintaining organismal health. The innate immune system is far older, and conserved in all forms of plant and animal life. The innate immune system has evolved to rapidly respond to pathogen associated molecular patterns (PAMPs) that are conserved among harmful microbes and pathogens. While the ability to recognize potential pathogens via PAMPs provides a means for a robust rapid response, the degree of coverage is limited to molecules and pathogens who contain PAMPs. To combat this, the adaptive immune system was evolved to recognize exquisitely specific “non-self” antigens and respond to them. This allows our adaptive immune system to recognize a far greater number of pathogenic organisms that are not recognized by the PAMP receptors in our innate immune cells. The adaptive immune response is far slower, however cells of the adaptive immune system, once activated can also sustain a population of cells that essentially remember the pathogen and can respond more rapidly upon a secondary exposure. Thus, our innate and adaptive immune systems synergize, providing immediate response from the innate immune cells and more sustained protection from adaptive immune cells.

CD4+ T cells, or T helper cells are integral in the control and regulation of inflammation. Their defining feature is the T cell receptor which enables activation in an antigen-specific manner. T helper cells secrete cytokines that promote either pro- or anti-inflammatory states and play an important role in the progression of numerous disease states. The balance between pro-inflammatory Th1 and Th17 cells and
anti-inflammatory T regulatory (T_{reg}) cells is critical for immune homeostasis.\textsuperscript{175, 180} T_{regs} cells help control inflammation in multiple diseases, by multiple mechanisms including secretion of anti-inflammatory cytokines which control the function of cytotoxic T cells, as well as innate immune cells such as macrophages. Additionally, they inhibit the growth and differentiation of pro-inflammatory T cells subtypes to further prevent inflammation. In cases of aberrant inflammation, there is typically and underrepresentation of T_{regs} response relative to T_{H1} and T_{H17}.\textsuperscript{176, 180-182}

5.1.2. Small molecule screening to identify novel pathways (DYRK1A, Khor et al.)
Small molecule therapeutics which specifically modulate the CD4+ T helper cell differentiation are lacking. A fuller understanding the pathways that control the direction of CD4+ differentiation is critical in identifying novel pathways for potential therapeutic intervention. Previous work from Khor et al.\textsuperscript{19} utilized an unbiased screen to identify small molecule regulators of T cells. Inhibitors of DYRK1A were discovered to regulate this balance, enhancing the differentiation of T_{regs} while blunting T_{H17} differentiation. These effects were evident both \textit{in vivo} and \textit{in vitro}, and capable of lowering inflammatory responses in disease models.

I recently determined that the Mediator associated kinase, CDK8 regulates the expression of IL-10 in primary bone marrow derived dendritic cells (BMDCs) (Manuscript under review at NCB), establishing an important role for CDK8 in innate immune activation. CDK8 has been shown to directly phosphorylate multiple transcription factors including S\textsuperscript{727} of STAT1 (in the transcriptional activation domain),\textsuperscript{11} and Ser\textsuperscript{206} of SMAD1/5 (in the linker region).\textsuperscript{12} These studies suggest that CDK8 has established
transcription factors as its substrates. CDK8 activity has been linked to activity on the immune system from modulation of the IFN\(\gamma\) STAT1 response\(^91\) to restraining NK cell activation\(^93\) to tumor promoting paracrine activity.\(^95\) Furthermore, a significant number of selective CDK8 inhibitors with varying pharmacological properties have been reported\(^72\-77,95,104\), allowing for exploration of \textit{in vitro} and \textit{in vivo} CDK8 driven biology. Given our previously established role for CDK8 in innate immune activation, we began to explore a possible role for CDK8 in regulation of adaptive immunity.

5.2. Results

5.2.1. CDK8 inhibition enhances the differentiation of T\textsubscript{reg} in \textit{vitro}

I established that CDK8 inhibition works to promote a tolerogenic programs in dendritic cells, increasing their secretion of IL-10 in response to various TLR ligands. Having identified that CDK8 has a pro-tolerogenic effect on dendritic cells during innate immune activation, we endeavored to determine if CDK8 inhibition had a pro-tolerogenic effect on differentiation of CD4+ T helper cells. Utilizing \textit{in vitro} systems developed by Bernard Khor in the Xavier Lab (MGH) we determined that CDK8 inhibition does in fact promote tolerogenic programs in \textit{in vitro} differentiation models for CD4+ T helper cells, results which are summarized in Figures 5.1 and 5.2.

Naïve CD4+/CD62L\textsuperscript{+} were differentiated utilizing suboptimal T\textsubscript{reg} (T\textsubscript{reg}\textsuperscript{Lo}, using low concentrations of TFG\(\beta\)) differentiation conditions in the presence or absence of a small molecule CDK8 inhibitor, DCA (Figure 5.1a). These were compared to established gold standard conditions for \textit{in vitro} differentiation of T\textsubscript{reg} (T\textsubscript{reg}\textsuperscript{Hi}, using high concentrations of TGF\(\beta\)). After four days, cells were collected and stained for high levels of the T\textsubscript{reg} hallmark
transcription factor FOXP3. To determine degree of $T_{\text{reg}}$ differentiation, we measured the percent of cells FOXP3+ cells in each condition using flow cytometry and calculated the fold change over $T_{\text{reg}}^{\text{Lo}}$ conditions with no inhibitor present (Figure 5.1b).

DCA, the highly selective CDK8 inhibitor, enhanced the differentiation of naïve CD4+ cells into $T_{\text{reg}}$ in a dose dependent manner, and produced a fractional enhancement of FOXP3+ above the no treatment condition similar to gold standard $T_{\text{reg}}^{\text{Hi}}$ conditions. This enhancement was conserved across a diverse set of CDK8 inhibitors, including the recently published CCT251921 and our previously described CDK8 inhibitor BRD6989 (Figure 5.1c). This effect is limited to inhibition of CDK8, as other transcriptional CDK inhibitors display no $T_{\text{reg}}$ enhancement (Figure 5.1d).
Figure 5.1: Determination of the effect of CDK inhibitors on differentiation of naïve CD4+ T cells to T<sub>regs</sub>. a) Naïve CD4+/CD62L+ T cells were isolated from Balb/c using magnetic separation, then cultured on αCD3/αCD28 coated plates in T<sub>reg</sub><sup>Lo</sup> conditions with or without inhibitor. b) After re-feeding with inhibitor and IL-2 on day 2, the %FOXP3+ cells were determined via Flow-cytometry on day 4 to quantitate the degree of differentiation into T<sub>regs</sub>. c) Inhibitors of CDK8 but not d) other CDKs (CDK4/6 inhibitor palbocyclib, CDK7 inhibitor THZ1, or CDK9 inhibitor NVP2) are selectively able to enhance differentiation of T<sub>regs</sub>. Additionally, the other CDK inhibitors do not show any appreciable enhancement at levels which are not cytotoxic. The slight enhancement that is seen at higher concentrations of Palbocyclib is due to an artifact of the in vitro experiment in which lower numbers of T cells tend become biased towards T<sub>regs</sub>. 
5.2.2. CDK8 inhibitors effect on pro-inflammatory CD4+ T cells

To determine whether these effects were relegated to a lineage specific enhancement of the anti-inflammatory Tregs as opposed to a general activation of CD4+ proliferation, we examined the effect of CDK8 inhibitors on the differentiation of the pro-inflammatory T\(_H1\) (Figure 5.2c) and T\(_H17\) (Figure 5.2d) lineages. Again, naïve CD4+/CD62L+ were grown in suboptimal T\(_H1\) (T\(_H1^{1\text{Lo}}\)) or T\(_H17\) (T\(_H17^{1\text{Lo}}\)) conditions to determine if CDK8 inhibitors enhance T\(_H1\) or T\(_H17\) differentiation (Figure 5.2a). These were compared to optimal T\(_H1\) (T\(_H1^{1\text{Hi}}\)) or T\(_H17\) (T\(_H17^{1\text{Hi}}\)) conditions. After four days, cells were re-stimulated with PMA and ionomycin, and treated with Golgi Stop™ to induce the production of cytokines then block their secretion, allowing for identification via intracellular staining. T\(_H1\) cells were then stained for IFN\(_\gamma\) and IL-10, and T\(_H17\) for IL17A, and the percentage of positive cells measured via flow cytometry.

These results showed that CDK8 inhibitors had no impact on either enhancement of T\(_H1^{1\text{Lo}}\) or T\(_H17^{1\text{Lo}}\) conditions or inhibition of T\(_H1^{1\text{Hi}}\) or T\(_H17^{1\text{Hi}}\) conditions, demonstrating that CDK8 inhibitors neither enhance nor inhibit pro-inflammatory T\(_H1\) & T\(_H17\) differentiation. Rather, they specifically enhance anti-inflammatory T\(_\text{reg}\) differentiation, highlighting a capacity to engage tolerogenic programs in both innate & adaptive immune cells.
Figure 5.2: Determination of effect of CDK8 inhibition on differentiation of pro-inflammatory CD4+ T cell lineages. a) Naïve CD4+/CD62L+ cells from Balb/c mice were isolated and differentiated under TH1 or TH17 conditions with or without inhibitor. On day four the cells were re-stimulated and the fraction of IFNγ+ (TH1) or IL-17+ (TH17) cells were determined via flow-cytometry. Fractional enhancement was determined in comparison to the DMSO treated control. b) CDK8 inhibitors have minimal to no impact on pro-inflammatory CD4+ T cell lineages. CDK8 inhibition showed no enhancement of TH1 differentiation under TH1 Lo conditions or suppression of TH1 Hi differentiation. c) Similarly, no enhancement of TH17 differentiation was seen under TH17 Lo conditions, with a potentially slight suppressive seen by CDK8 inhibition in TH17 Hi conditions.
5.2.3. CDK8 Inhibitor Enhanced T\textsubscript{regs} are Functionally Suppressive \textit{In Vitro}

Having identified CDK8 inhibition as a novel pathway for enhancing T\textsubscript{reg} differentiation \textit{in vitro}, I wanted to establish the functionality of the CDK8 inhibitor-enhanced \textit{in vitro} differentiated T\textsubscript{regs}. Functional T\textsubscript{regs} are capable of suppression of the division of CD4+ T cells.\textsuperscript{19} Naïve CD4+/CD62L+ cells were isolated from Foxp3\textsuperscript{IRES-GFP} mice and differentiated under T\textsubscript{reg}\textsuperscript{Lo}+DCA or gold standard T\textsubscript{reg}\textsuperscript{Hi} conditions as previously described.\textsuperscript{19} These cells will co-express both GFP and FOXP3 from the same mRNA transcript. This allows direct determination of the FOXP3 expression by examining the GFP levels, which allows sorting of live and functional T\textsubscript{regs} using Fluorescence Activated Cell Sorting (FACS). To obtain a purified T\textsubscript{reg} population, following four days of differentiation, we sorted for the GFP\textsuperscript{Hi}/CD4+ cell population to use in an \textit{in vitro} suppression assay. Additionally, a T\textsubscript{reg}\textsuperscript{Lo} alone population was used as a control to confirm a fractional enhancement of FOXP3+ as compared to T\textsubscript{reg}\textsuperscript{Lo}+DCA or gold standard T\textsubscript{reg}\textsuperscript{Hi} conditions. However, the T\textsubscript{reg}\textsuperscript{Lo} alone population was not used in the \textit{in vitro} suppression assay, as FACS sorting would still yield GFP\textsuperscript{Hi}/CD4+ cells that will behave like \textit{bona fida} T\textsubscript{regs}, just producing a lower number.

To test the suppressive capabilities of our T\textsubscript{reg}\textsuperscript{Lo}+DCA or gold standard T\textsubscript{reg}\textsuperscript{Hi}, our T\textsubscript{regs} were co-cultured with T\textsubscript{responder} (T\textsubscript{H}0) cells. The T\textsubscript{H}0 cells were naïve CD4+/CD62L+ isolated from CD45.1 mice, but not polarized with any cytokine differentiation cocktails. Additionally, CD45.1 mice express a variant of CD45 cell surface protein that allows delineation of the population of T\textsubscript{responder} cells from our T\textsubscript{regs} by staining for the presences of CD45.1. T\textsubscript{responder} cells were pre-stained with CFSE, a cell permeable fluorescent dye, which upon entering the cell is phosphorylated and retained. The CFSE dye is divided between the cytoplasm of subsequent daughter cells, allowing for determination of the
number of cellular divisions based on the intensity of the CFSE stain in the CD45.1 T
responder cell population (Figure 5.3a,b).

The T_{H0} T_{responder} cells were activated with αCD3/αCD28 activation beads and co-
cultured with a serial dilution of FOXP3^{IRES-GFP} T_{regs} (either T_{reg}^{Lo}+DCA or gold standard
T_{reg}^{Hi}). On day 3, the cells were collected and stained for CD45.1 to identify the naïve
T_{responder} cells, and analyzed via flow cytometry. The degree of CFSE in the CD45.1+
T_{responder} cells was analyzed with a histogram to identify the number of cell divisions
undergone based on the intensity of the stain. Decreased CFSE intensity correlates more
cellular divisions, resulting from CFSE division between the cytoplasm of subsequent
daughter cells. Increased cellular divisions indicate a lower suppressive capacity of the
T_{regs}. To determine how functionally suppressive our CDK8 inhibitor enhanced (T_{reg}^{Lo}
+DCA) T_{regs} were, we compared their suppressive ability to the gold standard T_{reg}^{Hi}
conditions (Figure 5.3c). From this we established that CDK8 inhibitor enhanced T_{reg} in
vitro suppressive capacity is identical to gold standard T_{reg}^{Hi} cells differentiated under high
TGFβ conditions.
Figure 5.3: *In vitro* suppression assay to determine the functionality of our CDK8 inhibitor-enhanced T\_regs. a) Naive CD4+/CD62L+ cells were isolated from mice expressing FOXP3\^{RES/GFP} and differentiated under T\_reg\^{Lo+DCA} or the gold standard T\_reg\^{Hi} conditions. On day four, CD4+/GFP\^{Hi} cells were sorted via FACS and used for the *in vitro* T\_reg suppression assay. T\_regs were co-cultured with CFSE labeled T\_responder cells isolated from CD45.1 mice. b) Histograms showing the intensity of CFSE labeled CD45.1 T\_responder cells without and with T\_regs. The suppressive capability of the T\_reg\^{Lo+DCA} or the gold standard T\_reg\^{Hi} was determined based on ability to limit the number of CD45.1 T\_responder cell divisions as determined via and histogram showing the intensity CFSE label in T\_responder cells. c) CDK8 inhibitor enhanced T\_reg are functionally suppressive *in vitro*, equivalent to our gold standard T\_reg\^{Hi} condition.
5.2.4. CDK8 inhibitor enhanced T_{regs} are functionally suppressive in vivo

While CDK8 inhibitor enhanced T_{regs} were functionally suppressive *in vitro*, we wanted to determine if these results extended to *in vivo* models of inflammation. To determine this, we used an established Type I diabetes inflammatory disease model, known to be dependent on T_{regs}. This model utilizes NOD/SCID mice, who lack T cells, and BDC2.5/FOXP3^{RES/GFP} mice derived naïve T cells, which have a T cell receptor that recognizes pancreatic beta cells. When naïve CD4+ T helper cells isolated from BDC2.5/FOXP3^{RES/GFP} mice are injected into NOD/SCID mice alone, these mice rapidly develop diabetes with in 5-10 days, as the inject T helper cells tend to differentiate into inflammatory T cell lineages thus specifically recognizing and ablate insulin producing pancreatic beta cells. However, if the naïve T cells are co-injected with functional T_{regs}, the onset of diabetes can be significantly delayed (*Figure 5.4a*).

Naïve CD4+/CD62L+ cells from BDC2.5/FOXP3^{RES/GFP} were isolated and differentiated over 4 days (both T_{reg}^{Lo}+DCA and T_{reg}^{Hi}), then FACS sorted for CD4+/GFP^{Hi} T_{regs}, and co-injected with naïve CD4+/CD62L+/GFP^{Lo} cells from BDC2.5/FOXP3^{RES/GFP} mice. Daily blood glucose measurements were taken, monitoring for onset of diabetes, defined as 2 concurrent days of glucose measurements >250 mg/dL. The CDK8 inhibitor enhanced T_{regs} delayed the onset of diabetes by two weeks, from Day 7 to around Day 21, which was equivalent or better to the gold standard T_{reg}^{Hi} condition (*Figure 5.4b*) Thus CDK8 inhibitor enhanced T_{regs} are functionally suppressive in both *in vitro* and *in vivo* conditions.
Figure 5.4: Determination of CDK8 inhibitor-enhanced T_{reg} functionality in an in vivo T_{reg} dependent model of inflammation. a) Naïve CD4+/CD62L+ cells were isolated from BDC2.5/FOXP3^{IRES/GFP} mice, which express a transgene for a T cell receptor that recognize pancreatic beta cells. These were cultured for four days under T_{reg}^{Hi} or T_{reg}^{Lo}+DCA conditions, then sorted to obtain CD4+/GFP^{Hi} T_{regs}. Additionally, naïve CD4+/CD62L+/GFP^{Lo} cells were isolated from BDC2.5/FOXP3^{IRES/GFP} mice. Naïve cells were injected into NOD/SCID mice, with or without T_{reg}^{Hi} or T_{reg}^{Lo}+DCA cells. b) Mice without functional T_{regs} (No T_{reg} condition) developed diabetes, as measured by glucose levels >250mg/dL, within one week. However, mice co-injected with either T_{reg}^{Hi} or T_{reg}^{Lo}+DCA cells had delayed onset of diabetes by 2 weeks. CDK8 inhibitor enhanced T_{regs} are functionally suppressive in vivo, at or superior to gold standard T_{reg}^{Hi} cells.
5.3. Discussion and Conclusions

5.3.1. CDK8 Inhibition Promotes Tolerance in Innate and Adaptive Immunity

Our previous work established a role for CDK8 in the regulation of innate immunity by enhancing IL-10 secretion in dendritic cells through a novel mechanism via the direct phosphorylation of AP1 by CDK8 in a regulatory domain. More recently we have begun to explore additional roles CDK8 may have in immune regulation, specifically identifying a role for CDK8 inhibition in enhancing differentiation of FOXP3+ T_{reg} cells.

We have shown that CDK8 inhibitors are capable of selectively enhancing the anti-inflammatory T_{reg} cells without no impact on differentiation of pro-inflammatory T_{H1} or T_{H17} cells. Moreover, CDK8 inhibitor enhanced T_{reg} cells are functional in in vitro and in vivo systems, comparable to gold standard T_{reg}^{Hi} cells. Previous small molecular enhancers of either IL-10 (SIK inhibitors)\textsuperscript{17,116} or T_{reg} cells (DYRK1A inhibitors)\textsuperscript{19} that have been identified in the Xavier lab have only showed functionality in one system, promoting tolerance in either the innate or adaptive immune system but not both. Interestingly, CDK8 appears capable of regulating tolerance in the both innate and adaptive immunity.

5.3.2. Potential Unifying Mechanism through AP1 Regulation

Our recent work identified a novel substrate for CDK8 in innate immune activation, via the direct phosphorylation of the negative regulatory S243 of c-Jun, a subunit of AP1. This finding was key to identification of the mechanistic reason for CDK8 inhibitor driven enhancement of IL-10 via AP1. Intriguingly AP1 is an established regulator of T cell differentiation, thus this insight may provide a connection between CDK8’s ability to
promote tolerance in both innate and adaptive immunity.\textsuperscript{159, 183} More work is needed to fully elucidate the mechanism behind these observations.

These findings suggest that targeting CDK8 could provide a novel therapeutic method for regulating diseases with aberrant inflammation by targeting both innate and adaptive immunity. Efforts are underway to explore this using various T\textsubscript{reg} dependent models of inflammation and the highly selective CDK8 inhibitor DCA, provided in collaboration with Phil Baran’s Lab at Scripps Research Institute.\textsuperscript{103, 184}

5.4. Materials and Methods

Mice, antibodies and reagents. Balb/c, C57Bl/6, Cd45.1+/+, Rag1\textsuperscript{−}/−, Foxp3IRES-GFP, Il17IRES-GFP, NOD-scid and NOD-BDC2.5 mice were obtained from Jackson Labs. NOD-BDC2.5.Foxp3IRES-GFP mice were obtained from the JDRF Transgenic Core (Harvard Medical School, Boston, MA).

Murine T cell isolation and culture. Naïve T cells (CD4+CD62L\textsuperscript{+} double positive) were isolated using CD4 negative enrichment kits (Stemcell Technologies, Vancouver, Canada) and CD62L microbeads (Miltenyi Biotec, San Diego, CA). Purity was confirmed to be >95% pure by flow cytometry. Cells were cultured on 96-well plates pre-coated with anti-CD3 and anti-CD28. T\textsubscript{reg} and T\textsubscript{H1} cultures were fed with equal volume of IL-2-supplemented media (10 ng/ml) and retreated with compound at day 2, split 1:2 into IL-2-supplemented media at day 3 and analyzed at day 4. T\textsubscript{H17} cultures were treated similarly however, no IL-2 was added with in subsequent feeds. Cellular divisions were monitored using CFSE (Life Technologies, Carlsbad, CA) per manufacturer’s instructions. Cytokine conditions for differentiation are as listed in below table 5.1:
Table 5.1: CD4+ T cell cytokine conditions for differentiation.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Neutralizing antibodies</th>
<th>Cytokines (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T&lt;sub&gt;reg&lt;/sub&gt;&lt;sup&gt;hi&lt;/sup&gt;</td>
<td>Anti-IL-12</td>
<td>TGFβ (10)</td>
</tr>
<tr>
<td></td>
<td>Anti-IFNγ</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Anti-IL-4</td>
<td></td>
</tr>
<tr>
<td>T&lt;sub&gt;reg&lt;/sub&gt;&lt;sup&gt;low&lt;/sup&gt;</td>
<td>Anti-IL-12</td>
<td>TGFβ (1.5)</td>
</tr>
<tr>
<td></td>
<td>Anti-IFNγ</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Anti-IL-4</td>
<td></td>
</tr>
<tr>
<td>T&lt;sub&gt;H17&lt;/sub&gt;&lt;sup&gt;hi&lt;/sup&gt;</td>
<td>Anti-IL-12</td>
<td>TGFβ (0.5)</td>
</tr>
<tr>
<td></td>
<td>Anti-IFNγ</td>
<td>IL-6 (20)</td>
</tr>
<tr>
<td></td>
<td>Anti-IL-4</td>
<td>IL-1β (20)</td>
</tr>
<tr>
<td>T&lt;sub&gt;H17&lt;/sub&gt;&lt;sup&gt;low&lt;/sup&gt;</td>
<td>Anti-IL-12</td>
<td>TGFβ (0.5)</td>
</tr>
<tr>
<td></td>
<td>Anti-IFNγ</td>
<td>IL-6 (5)</td>
</tr>
<tr>
<td></td>
<td>Anti-IL-4</td>
<td></td>
</tr>
<tr>
<td>T&lt;sub&gt;H1&lt;/sub&gt;&lt;sup&gt;hi&lt;/sup&gt;</td>
<td>Anti-IL-4</td>
<td>IL-12 (10)</td>
</tr>
<tr>
<td>T&lt;sub&gt;H1&lt;/sub&gt;&lt;sup&gt;low&lt;/sup&gt;</td>
<td>Anti-IL-4</td>
<td>IL-12 (0.05)</td>
</tr>
</tbody>
</table>

**Flow cytometry.** 4 hr prior to analysis, T<sub>H1</sub> and T<sub>H17</sub> cultures were treated with PMA and ionomycin (50 and 500 ng/ml respectively, Sigma Aldrich, St. Louis, MO) in the presence of Golgistop (BD Biosciences, San Jose, CA), to re-stimulate and allow for production of cytokines for staining. Cells were stained with LIVE/DEAD and anti-CD4-FITC before being fixed and permeabilized using either Foxp3 fixation/permeabilization buffers (eBioscience, San Diego, CA) or Phosflow Fix/Perm buffers (BD Biosciences, San Jose, CA) as indicated. Intracellular staining was performed per manufacturer’s instructions. Counting beads (10 μm, Spherotech, Lake Forest, IL) were added at 5000 per well. Acquisition was performed on a FACSVerse (BD Biosciences, San Jose, CA) and analyzed using FlowJo software (Treestar, Ashland, OR). Fractional enhancement was determined by increase in percentage lineage-committed cells, relative to maximal
cytokine-driven enhancement. Fractional inhibition was calculated relative to percentage lineage-committed cells treated with DMSO. Cell sorting was performed on a FACS Vantage (BD Biosciences, San Jose, CA)

**In vitro Treg suppression assay.** This was performed as previously described (Collison and Vignali, 2011). Briefly, naïve CD45.1 T<sub>responder</sub> cells (CD45.1+CD4+CD62L+) cells were isolated and labeled with CFSE (Invitrogen, Grand Island, NY) per manufacturer’s protocol, plated at 5 x 104 cells per well in 96-well U bottom plates and co-cultured with sorted CD45.2+Foxp3IRES-GFP T<sub>reg</sub> cells at ratios indicated in the presence of anti-CD3/28 beads (Dynabead, Grand Island, NY) and analyzed by flow cytometry 3 days later.

**Treg suppression—T1D model.** As previously described, 5X10<sup>4</sup> CD4+CD62L+ T cells isolated from NOD-BDC2.5 mice were administered intravenously to NOD-scid mice with or without 1X10<sup>5</sup> sorted T<sub>reg</sub> cells generated from NOD-BDC2.5.Foxp3 IRES-GFP mice (Herman et al., 2004; Tarbell et al., 2004). Blood glucose levels were measured with a handheld Contour glucometer (Bayer, Leverkusen, Germany) at days 3, 6, 8 and every day thereafter. Diabetes was diagnosed when blood sugar was over 250 mg/dl for 2 consecutive days.

**Histology.** Tissues were preserved in 10% formalin. Paraffin embedding, sectioning and staining with either hematoxylin and eosin or Periodic acid-Schiff/Alcian Blue were performed by the Histopathology Research Core (Massachusetts General Hospital, Boston, MA)

**Study approval.** All experiments were performed with the approval of the IACUC of Massachusetts General Hospital (Boston, MA).
Chapter 6: Conclusion
6.1. CDK8 as a regulator of transcription via phosphorylation of specific transcription factors

The transcriptional co-activator Mediator, found primarily at cis-acting regulatory elements known as enhancers, is essential for the transcription of almost all protein coding genes in eukaryotes. Mediator exists in two main forms, core Mediator, and CDK8-Mediator, which possess different functions. \(^78\) siRNA screening has identified CDK8 loss as a negative regulator of WNT \(\beta\)-catenin signaling in colorectal cancer cells. \(^7-9\) Additionally, high levels of expression of CDK8 in gastric adenocarcinoma was correlated with a poor patient prognosis. \(^10\) Thus CDK8 was investigated for its oncogenic potential, leading to development of multiple selective small molecule inhibitors of CDK8. \(^74, 75, 77, 95, 104\)

\(^104\) Work from the Shair lab supported CDK8 as an oncogenic target, showing a strong anti-proliferative effect in a subset of AML cells. In these AML lines, CDK8 inhibition increases gene expression of super enhancers leading to cell death. \(^104\)

However, recent research suggests CDK8 does not function as a canonical oncogene. In fact, CDK8 can function as a tumor suppressor in different tumor lineages such as endometrial cancer \(^101\), and TAL \(^94\). Insight to these observations may come from a divergence in the function of CDK8 structural role as compared to its kinase activity. Deletion of CDK8 or its submodule leads to impairment of transcription and is embryonically lethal. \(^84, 85, 58\) However, there is an emerging role for CDK8 in the control of select transcriptional networks, often via direct phosphorylation of gene specific transcription factors either activating or repressing their function including STAT1 \(^11\), SMADs \(^12\), E2F1 \(^13\), HIF1a \(^14\), p53 \(^15\), serums response network. \(^7, 9, 14-16, 62, 89-92, 94, 105, 156, 185\)
My initial studies suggest that, while loss of CDK8 may be anti-proliferative, CDK8 kinase inhibition is not. Indeed, CDK8 inhibition showed no anti-proliferative impact on the subset colorectal cells previously reported as sensitive to CDK8 knock down with siRNA. Instead, my work highlighted CDK8’s control over a subset of genes induced by IFNγ activation through modulation of STAT1 S727 phosphorylation, an established CDK8 target. Additionally, we determined that CDK8 inhibition enhances IL-10 production in activated myeloid cells, which occurs through CDK8 phosphorylation of the transcription factor AP1. CDK8 phosphorylates c-Jun, a subunit of AP1, at S243 which is established negative regulatory site. This supports a distinct role for CDK8 catalytic activity in transcriptional regulation, where in kinase activity is critical for cellular response to external stimuli.

6.2. CDK8 as a regulator of tolerance in both innate and adaptive immunity

Enhancing production of the anti-inflammatory cytokine IL-10 is a promising strategy to suppress pathogenic inflammation. IL-10 enhancement has shown clinical promise but is, as of yet, unsuccessful. We conducted an unbiased phenotypic screen for small molecules that enhance IL-10 production in activated dendritic cells in order to elucidate novel pathways involved in regulating IL-10. Previous studies have established SIK inhibition and other cAMP enhancers as a target for enhancement of IL-10. However, some of the available cAMP enhancers exhibit unwanted toxicity which may limit therapeutic potential. Mechanism-of-action studies with the prioritized screening hit (BRD6989) identified the Mediator-associated kinase CDK8, and its paralog CDK19, as negative regulators of IL-10 production during innate immune activation. This effect was associated with reduced phosphorylation of a negative
regulatory site on c-Jun, suggesting CDK8 driven regulation of innate immune activation occurs through modulation of phosphorylation of c-Jun thus impacting its transcriptional activity.

While the innate immune system is important for rapid response to pathogens via recognition of PAMPs, it only represents a portion of our immune system. Our adaptive immune system allows for detection of a wider range of "non-self" antigens, creating a broader degree of protection from foreign pathogens. CD4+ T helper cells are integral in controlling the function of both innate and adaptive immune cells by secreting cytokines which activate phagocytic cells, control growth of cytotoxic T cells, and assist in B cell class switching among other functions.

However, unlike myeloid cells, CD4+ T helper cells terminally differentiate into either a pro-inflammatory or anti-inflammatory lineage, which delineate their function. This balance between the differentiation of naïve CD4+ T helper cells between pro-inflammatory T\textsubscript{H}1 and T\textsubscript{H}17 cells and anti-inflammatory T regulatory (T\textsubscript{reg}) cells is critical for immune homeostasis. In multiple inflammatory diseases there is an abundance of pro-inflammatory T\textsubscript{H}1 and T\textsubscript{H}17 cells with an underrepresentation of anti-inflammatory T\textsubscript{reg}. Elucidation of the pathways which regulate CD4+ differentiation into either pro- or anti-inflammatory states may identify new areas for therapeutic intervention in inflammatory diseases.

Consequently, I determined that CDK8 inhibition enhances differentiation of naïve CD4+/CD62L+ cells towards anti-inflammatory T\textsubscript{reg} without enhancing differentiation of pro-inflammatory T\textsubscript{H}1, and T\textsubscript{H}17 cells. Furthermore, CDK8 inhibitor enhanced T\textsubscript{reg} are
functional in both *in vitro* and *in vivo* models. Thus, CDK8 inhibition may be a novel and interesting target for promotion of tolerance in both innate and adaptive immunity.

### 6.3. Promise of CDK8 as a therapeutic target

Growing interest in Mediator-associated kinases as therapeutic targets in cancer spurred development of several chemically distinct CDK8/19 inhibitors. These include the potent dual CDK8/19 inhibitor CCT251921\(^{151}\), and the natural product Cortistatin A (CA) and a close analog (DCA)\(^{105,149}\). However, emerging research indicates that sustained CDK8 inhibition may be toxic *in vivo*.\(^{73}\) Given that CDK8/19 are fundamental regulators of transcription in numerous cell types, it is not surprising that small-molecule inhibitors of these Mediator-associated kinases were poorly tolerated *in vivo* at doses that achieve the sustained target occupancy required for anti-tumor activity.\(^{73}\) Additionally, increased IL-10 expression and suppression of anti-tumor immune responses have well-established deleterious impact on tumor progression and chemotherapy resistance\(^{164-166}\). Thus CDK8/19 inhibition driven anti-inflammatory effects will warrant examination prior to employment of CDK8/19 inhibitors therapeutically in cancer.

These toxicities may preclude therapeutic targeting of CDK8/19. However, it is possible that the anti-inflammatory consequences of CDK8/19 inhibition is achievable *in vivo* using altered dosing regimens. Our data suggest that inhibiting CDK8/19 affects a limited number of pathways in myeloid cells including several targeted by clinically approved inhibitors of Janus kinases and the proteasome, respectively.\(^{167,168}\) However, it is not yet clear if use of CDK8 inhibitors to upregulate IL-10 will be a functional and tolerated treatment for inflammatory disorders.
Importantly, we found that both CCT251921 and DCA, like BRD6989, promote IL-10 production in R848-stimulated dendritic cells in a manner that depends on an intact Cyclin C/CDK8 complex. Clinical observations that targeted delivery of IL-10 to inflamed tissues suppresses inflammation suggest that use of this strategy with small molecules may hold promise.\textsuperscript{141, 142} Notably, CDK8 selectively upregulates IL-10 production by activated myeloid cells suggesting its immunomodulatory activity could be localized to sites of inflammation circumventing dose-limiting toxicities associated with systemic delivery of IL-10.\textsuperscript{162, 163} Still, a thorough investigation of potential toxicities at dose of CDK8/19 inhibitors associated with immunomodulatory effects \textit{in vivo} will be a critical for determining therapeutics potential of targeting Mediator-associated kinases.
7. Appendix

7.1. Design of CDK11 inhibitors for osteosarcoma

7.1.1. Identification of scaffolds with CDK11 inhibition

CDK11 is a member of the Cyclin dependent kinase family, and appears to be involved in regulation of RNA splicing. Collaborators in a group at Massachusetts General Hospital determined that osteosarcoma lines expressing high levels of CDK11 were sensitive to CDK11 knock down via siRNA. Thus, we endeavored to identify selective CDK11 inhibitors that may have had therapeutic potential in the treatment of osteosarcoma. Previous small molecule profiling efforts in the Gray lab had identified subsets of small molecule kinase inhibitors which purportedly bound CDK11. This allowed for a starting point to identify potential small molecule inhibitors of CDK11.

CDK11 is a large kinase, around 110 kDa, with multiple potentially active isoforms. Additionally, it as part of a large protein complex in the spliceosome which can be technically challenging to reconstitute ex vivo. Moreover, as there were no reported CDK11 substrates at the time there was no easy way to measure the activity of potential CDK11 inhibitors in cells. 188

7.1.2. Identification of compounds which bind CDK11

I designed agarose beads which had the Pan-CDK inhibitor, AT7519 immobilized on the surface (Figure 7.1a.). 189 AT7519 has a free NH2 group which allowed for covalent linkage to NHS activated agarose beads. These beads can pull down CDK11 from cell lysate of high CDK11 osteosarcoma expressing lines, KHOS and U2OS. Additionally, CDK2 and CDK11 binding was selectively competed off with free AT7519, as monitored via western (Figure7.1b), confirming their ability to pull down members of the CDK family.
Thus these beads provide a method to interrogate CDK11 binding in a low throughput manner. The potential CDK11 inhibitors were tested for their ability to compete off CDK11 binding, alighting on a subset of inhibitors which appeared to compete off CDK11 in a dose dependent manner Figure 7.1c. From there, these small molecules were tested on osteocarcenoma lines that were sensitive to CDK11 siRNA. Some of these identified CDK11, such as JWD047, binders showed promising efficacy of CDK11 high expressing U2OS and KHOS cells. However, while lower, their anti-proliferative impact against low CDK11 expressing CS-1 osteosarcoma cells was still significant. Since these inhibitors inhibited multiple off target kinases, it was not possible to determine if the cellular activity was due to inhibition of CDK11 (Figure 7.1 d).
Figure 7.1: Development and characterization of potential CDK11 inhibitors using a pan-CDK inhibitor. a) Structure of the Pan CDK inhibitor AT7519. B) Confirmation of ability to pull down CDK2 and CDK11 with AT7519 immobilized on beads. Unlabeled beads are unable to pull down CDK2 or CDK11. However, addition of AT7519 to beads sees effective pull down of both CDK2 and CDK11. Furthermore, addition of free AT7519 leads to competition of CDK2 and CDK11 as monitored by disappearance of those bands via western. C) Dose dependent competition of CDK11 from beads with potential CDK11 inhibitors. D) Cellular viability assay for efficacy of JWD047 on CDK11 high expressing U2OS and KHOS cells and low CDK11 CS-1 cells. While by IC50 JWD047 seems most potent on the high CDK11 expressing cells, there is significant effect on CS-1 cells.
7.1.3. Conclusions

Over all, due to the lack of either a robust biochemical assay or clear cellular target or phenotype, there were too many challenges to identify a robust and selective CDK11 inhibitor. However, it is possible with new and emerging literature, that CDK11 may be targetable through other means.

7.1.4. Materials and Methods

Development of AT7519 linked agarose beads. AT7519 was coupled to a 10-atom hydrophilic spacer arm attached to a solid phase agarose beads (12-16 µmol of carboxyl group/mL of drained matrix). Coupling was performed in DMSO overnight at room temperature with 3 equivalence of EDC or HATU for activation, 3 equivalence of basic amine (TIEA), and 1:10 of AT7519:Matrix. Reaction was monitored for the disappearance of free AT7519 via mass spectrometry. Following this, the beads were washed 3X with DMSO then, equilibrated with distilled water, and blocked with ethanolamine, according to the manufacture protocol, then wash three times in the Pierce IP lysis buffer plus 0.05% NaN3 for storage.

Immunoprecipitation of CDK11. Osteosarcoma cells which highly expressed CDK11 (U2OS and KHOS) were grown in DMEM with 10% FBS and Penicillin/Streptomycin supplemented. For pull downs, a 15 cm² dish was grown to 70% confluence. Cells were washed with ice cold PBS, then scraped on ice. Cells were pelleted by centrifugation, 5 minutes at 300XG, 4C. Then cells were lysed at 5-10X10^6 cells/mL in Pierce IP lysis buffer supplemented with 10mM MgCl₂ for 1 hr at 4C. Lysates were clarified by centrifuging at 15000G for 10 minutes and protein concentration determined via Pierce BCA protein quantification. Lysates for pull down were used at 1-5mg/mL of protein.
250uL of clarified lysate was pre-incubated on ice for 30 minutes with inhibitor at desired concentration, using 10µM AT7519 as a positive control for competition, and DMSO for total pull down. 25uL of dry bead volume was used for each sample with 250uL of clarified lysate, and was incubated in the cold room at 4C for 2 hours on a rotator. Then beads were pelleted at 300XG 5 minutes at 4C and washed 3X with 500uL of IP Lysis buffer, pelleting between. Remaining bound protein was released from the beads using LiDS containing sample buffer supplemented with beta mercapto ethanol, boiling at 90C for 10 minutes.

Samples were then chilled on ice for 10 minutes and CDK11 content analyzed via Western Blotting. In brief, samples were run on a 4-12% acrylamide gel (Invitrogen®) with MOPS running buffer (Invitrogen®), and transferred to nitrocellulose membrane. Membranes were blocked using non-fat milk in TBS +0.05% Tween-20 (TBST). Membranes were then washed 3X times and incubated with primary antibody for CDK2, to validate bead efficacy, and CDK11 (Sc-928, then later Cell Signaling #5524) in TBST with 2% BSA, and visualized using secondary conjugated to HRP on film. Additionally, total protein was visualized using silver stained gels.

**Cell viability assay.** U2OS, KHOS, CS-1 were grown in DMEM supplement with 10%FBS and Pen/Strep. When cells were at 70% confluence they were harvested via trypsinization, pelleted and counted. Cells were seeded in a 96-well dishes at 50,000 cells/mL and 100uL per well. Plates were spun down at 300XG for five minutes to promote even adherence, then treated with compound in a 12 point dose, in triplicate, for 48-72 hrs. Viability was measured using Cell Titer Glo® per manufacture protocol.
7.2. Development of Selective Covalent JAK3 Inhibitors

Li Tan, Koshi Akahane, Randall McNally, Kathleen Reyskens, Scott B. Ficarro, Suhu Liu, Micheal Pattision, Liv Johannesen, David A. Frank, Jarrod A. Marto, Thomas A. Look, Simon J. Arthur, Michael J. Eck, Nathanael S. Gray

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Jak3 is a member of the Janus Kinase family. The Jak family is integral in regulation of the STAT transcription factor family, a highly important transcription factor group involved in diverse cellular processes from immune activation to erythropoiesis. Jak3 has been implicated in multiple cancers, thus looked to be a promising potential therapeutic target. However, previous Jak family inhibitors lacked selectivity across the family leading to dose limiting toxicity which prevent clinical use. To potentially avoid this, a chemist Tan Li in the Gray lab began design and development of covalent Jak3 inhibitors which targeted a non-conserved Cysteine909 residue outside the pocket of Jak3, thus allowing potent inhibition of Jak3 without inhibition of related family members and the associated toxicity.

The results of these medicinal chemistry efforts were published in the Journal of Medicinal Chemistry in August 2015. To aid in this effort, I profiled the effects of these compounds on cellular viability and on their ability to inhibit downstream phosphorylation of target substrates of JAK3 (see Figures 7.8 and 7.9). Additionally, I provided edits to the manuscript through the review process. The manuscript is as follows:
7.2.1. Abstract
The Janus Kinases (JAKs) and their downstream effectors Signal Transducer and Activator of Transcription proteins (STATs) form a critical immune cell signaling circuit, which is of fundamental importance in innate immunity, inflammation and hematopoiesis and dysregulation is frequently observed in immune disease and cancer. The high degree of structural conservation of the JAK ATP binding pockets has posed a considerable challenge to medicinal chemists seeking to develop highly selective inhibitors as pharmacological probes and as clinical drugs. Here we report the discovery and optimization of 2,4-substituted pyrimidines as covalent JAK3 inhibitors that exploit a unique cysteine (Cys909) residue in JAK3. Investigation of structure-activity-relationship (SAR) utilizing biochemical and transformed Ba/F3 cellular assays resulted in identification of potent and selective inhibitors such as compounds 9 and 45. A 2.9 Å co-crystal structure of JAK3 in complex with 9 confirms the covalent interaction. Compound 9 exhibited decent pharmacokinetic properties and is suitable for use in vivo. These inhibitors provide a set of useful tools to pharmacologically interrogate JAK3-dependent biology.

7.2.2. Introduction
The Janus Kinases (JAKs) and their downstream effectors Signal Transducer and Activator of Transcription proteins (STATs) form a critical immune cell signaling circuit. There are four JAK family members in mammals, JAK1, JAK2, JAK3 and TYK2, each of which can bind to distinct cytokine and/or growth factor receptors. As the receptors that act upstream of JAKs are dimeric or multimeric, more than one JAK
molecule is recruited to each receptor. Ligand binding to the receptor results in a
conformation change that brings two JAK molecules close together allowing them to
trans-phosphorylate and activate each other. Once active, JAKs catalyze the
phosphorylation of tyrosine residues in the receptor allowing the recruitment of specific
STATs which are in turn phosphorylated by the JAK. This promotes STAT dimerization
which induces translocation to the nucleus and activation of gene regulatory programs.\textsuperscript{192, 193} JAK/STAT signaling is of fundamental importance in innate immunity, inflammation
and hematopoiesis and dysregulation is frequently observed in immune disease and
cancer.\textsuperscript{112, 114, 115, 194, 195} The JAKs have been the subject of extensive drug discovery
efforts and numerous small molecule ATP-competitive inhibitors have been developed\textsuperscript{196-200} including ruxolitinib (1, JAK1/2 inhibitor)\textsuperscript{201} approved as an anti-myelofibrosis drug,
tofacitinib (2, pan-JAK inhibitor)\textsuperscript{202} approved for rheumatoid arthritis, and several others
that are currently undergoing clinical investigation. The high degree of structural
conservation of the JAK ATP binding pockets has posed a considerable challenge to
medicinal chemists seeking to develop highly selective inhibitors as pharmacological
probes and as clinical drugs.\textsuperscript{203, 204, 205}

Among the JAK family JAK3 exhibits predominant expression in the hematopoietic
system in contrast to other JAKs which are broadly expressed in a variety of cell types.\textsuperscript{193} JAK3 is required for signaling by cytokines, including IL-2, IL-4, IL7, IL-9, IL-15 and IL-21,
that act via receptors that contain the common gamma chain ($\gamma_c$) cytokine receptor
subunit.\textsuperscript{206} In addition to the $\gamma_c$ subunit, most of these receptors contain a second unique
$\alpha$ subunit that determines cytokine specificity. In these receptors, JAK3 is binds to the $\gamma_c$
subunit and JAK1 to the $\alpha$ subunit. The IL-2R and IL-15R are exceptions to this as they
can from trimeric complexes through the recruitment of α and β chains. In this case the β subunit binds JAK1 and the α subunit, while involved in cytokine binding, does not directly bind to a JAK.\textsuperscript{207} Signaling via the γ\textsubscript{c} receptors is essential for the normal development and function of the immune system. Loss-of-function mutations in either γ\textsubscript{c} or JAK3 in human results in severe combined immunodeficiency caused by defective development of T- and NK cells. The role for JAK3 in T and NK cell development was subsequently confirmed by deletion of Jak3 in mice; JAK3 knockouts also lacked normal numbers of B cells indicating a more important role for JAK3 in B cell development in mice than humans.\textsuperscript{193, 208} On the basis of JAK3’s essential function in immune signaling and its restricted expression in hematopoietic tissues, JAK3 has been pursued as a target for the treatment of autoimmune and inflammatory diseases. JAK3 has also been explored as a potential anti-cancer target due to aberrant activation in several lymphoproliferative disorders;\textsuperscript{209} for example, mutations in JAK3 have been found in leukemias such as T-ALL and T-PLL.\textsuperscript{210} The A572V activating mutation in the kinase domain of JAK3 was discovered in acute megakaryoblastic leukemia and natural killer/T-cell lymphoma, and has been demonstrated to be capable of forming tumors in mice.\textsuperscript{211,212}

The most obvious strategy to develop small-molecule JAK3 inhibitors is to target the catalytic ATP-binding site of the JAK3 kinase domain (JH1). Numerous ATP-competitive JAK3 inhibitors have been developed (Figure 7.2).\textsuperscript{213-221} Tofacitinib (Compound 2) was developed as a specific JAK3 inhibitor to prevent organ-transplant rejection but subsequent studies revealed that it is also a potent inhibitor of JAK1 and JAK2.\textsuperscript{202, 222, 223} The combination of compound 2’s excellent potency, selectivity and
pharmacological properties have made it a favored inhibitor to interrogate JAK kinase activity in numerous biological models. In our efforts to develop a more selective Jak3 inhibitor, we noted that among JAK family members JAK3 is unique in having a cysteine residue at the gatekeeper-plus-7 (GK+7) position. This residue is Cys909 in human JAK, and it is structurally equivalent to cysteine residues in EGFR and BTK that have been successfully targeted by covalent kinase inhibitors that are now approved drugs. Afatinib\textsuperscript{226} (6) targets Cys797 in EGFR and ibrutinib\textsuperscript{227} (7) targets Cys481 in BTK. We and others have also developed mutant-selective inhibitors of the drug-resistant EGFR T790M that react covalently with Cys797. WZ4002\textsuperscript{228} (8) is a pyrimidine-based inhibitor with an acrylamide “warhead” that potently inhibits EGFR T790M. Recently two other groups have also reported the development of covalent JAK3 inhibitors. Taunton et.al reported the development of ‘reversible-covalent’ JAK3 inhibitors incorporating a cyanoacrylamide warhead which inhibited JAK3 in a biochemical kinase assay with an IC\textsubscript{50} of 100 nM while sparing other JAKs up to a concentration of 10 µM.\textsuperscript{229} Goedken et.al reported several tricyclic covalent JAK3 inhibitors with optimized inhibitors exhibiting biochemical IC\textsubscript{50}’s of 7 nM, good kinome selectivity and confirmed covalent binding to Cys909 using kinetic and mass-spectrometry studies.\textsuperscript{230} Here we report a medicinal campaign to derive potent disubstituted pyrimidine-based inhibitors that exploit an acrylamide electrophile to form a covalent bond to Cys909. Optimization was guided by cellular assays using Tel-JAK\textsuperscript{231} fusion kinase transformed Ba/F3 cells that provided an efficient and reliable measure of the ability of compounds to inhibit JAK signaling in cellular context.
7.2.3. Results

Amongst the 11 kinases that possess a cysteine at the GK+7 position only JAK3 and MAP2K7 have a methionine gatekeeper, which is also present in the drug-resistant EGFR\textsuperscript{T790M} that is targeted by the pyrimidine-based inhibitor WZ4002 (8). Compound 8 is a much less potent inhibitor of cellular JAK3 kinase activity (JAK3 \(K_d = 150\) nM, TEL-JAK3 Ba/F3 IC\textsubscript{50} = 2.82 \(\mu\)M) compared to T790M EGFR (EGFR\textsuperscript{L858R/T790M} Ba/F3 IC\textsubscript{50} = 8 nM).\textsuperscript{228} Through the syntheses of a small collection of analogs of 8 we discovered that relatively subtle changes could result in a dramatic improvement in JAK3 inhibitory potency relative to EGFR\textsuperscript{T790M}. For example, 9 where the ether linkage of 8 is replaced with an aminomethylene (-NHCH\textsubscript{2}-) linkage resulted in a great improvement in JAK3 inhibition in a fixed time-point Z’-lyte enzymatic assay (Life Technology, SelectScreen, IC\textsubscript{50} = 4.8 nM) (Scheme 7.1A).
To broadly assess the kinase selectivity, 9 was profiled against a diverse panel of 456 kinases (DiscoveRX, KinomeScan\textsuperscript{1,31}) using an \textit{in vitro} ATP-site competition binding assay at a concentration of 1.0 μM. Compound 9 exhibited good overall kinase selectivity with an S(5) selectivity score, defined as the percentage of kinases with scores less than 5 (S(5))\textsuperscript{31}, of 0.02. The results suggested that 9 most potently inhibits JAK3 and identified FLT3 and several TEC-family kinases as being potential off-targets (Figure 7.3). Enzymatic assays using the Z'-lyte or LanthaScreen\textsuperscript{33} formats confirmed enzymatic inhibition of FLT3 (IC\textsubscript{50} = 13 nM), TTK (IC\textsubscript{50} = 49 nM), BLK (IC\textsubscript{50} = 157 nM) and TXK (IC\textsubscript{50} = 36 nM). Compound 9 showed very low inhibition scores for other JAKs and wild-type (WT) EGFR, which is consistent with the over 180-fold higher IC\textsubscript{50}s against JAK1, JAK2, TYK2 and EGFR\textsuperscript{WT} (IC\textsubscript{50}s = 896, 1050, > 10000 and 409 nM respectively).
Figure 7.3: KinomeScan kinase selectivity profiles for Compound 9. Compound 9 were profiled at a concentration of 1 µM against a diverse panel of more than 456 kinases and mutants. Scores for primary screen hits are reported as a percent of the DMSO control (% control). The lower the Kd is likely to be, such that scores of zero represent strong hits. Scores are related to the probability of a hit but are not strictly an affinity measurement.

As enzymatic potencies sometimes do not translate into cellular inhibition, the ability of 9 to inhibit the proliferation of kinase-transformed Ba/F3 cells was evaluated. Ba/F3 cells are a murine pre-B cell that can readily be transformed with activated kinases to allow for growth in the absence of IL-3, and are frequently used to evaluate the activity of compounds against kinases of interest in a cellular context. We generated JAK1, JAK2 and JAK3 dependent Ba/F3 cell lines, where the JH1 domain of JAKs was fused with the oligomerization domain of Translocation ETS Leukemia protein (TEL) which results in constitutive tyrosine kinase activity and confers IL-3 independent proliferation. We had also engineered a TYK2 Ba/F3 cell line whose proliferation is driven by a TYK2<sup>E957D</sup> activating mutation. As further controls, we also tested a TEL-ABL and parental Ba/F3 cells grown in the presence of IL-3. To enable a direct comparison with the commonly used JAK inhibitors we profiled reported compounds 1-5, and 12 against this panel of Ba/F3 cells. Compound 1 exhibited the
most potent inhibition of JAK1 and JAK2 Ba/F3 cells, 2 exhibited most potent inhibition of JAK3 Ba/F3 cells and 3 exhibited most selective inhibition of JAK3. Overall the potency and selectivity of these inhibitors are consistent with their reported properties. Consistent with the biochemical assays, 9 selectively inhibited the proliferation of JAK3-dependent Ba/F3 cells (IC\textsubscript{50} = 60 nM) relative to other JAKs-dependent Ba/F3 cells, for which there was no antiproliferative effect at concentrations below 3.0 µM. The general antiproliferative activity that appears at concentrations of approximately 3.0 µM could be due to inhibition of other kinases such as TTK (aka Mps1, IC\textsubscript{50} = 49 nM) as inhibition of this kinase has been reported to decrease cancer cell viability\textsuperscript{236}.

To investigate the structural basis for achieving selectivity for JAK3 we solved the co-crystal structure of the JAK3 kinase domain in complex with 9 at resolution of 2.9 Å (Figure 7.4). In this structure (PDB ID 4Z16), the anilinopyrimidine moiety of 9 makes the expected bidentate hinge hydrogen bonds with Leu905, and continuous electron density is observed between the acrylamide warhead and Cys909, indicative of covalent bond formation. The kinase exhibits an active conformation, with the DFG-motif in the inward position and both tyrosines 980 and 981 in the activation loop phosphorylated. The orientation and interactions of the anilinopyrimidine portion of the compound closely resembles the interactions observed in the 8-EGFR\textsuperscript{T790M} co-structure (PDB 3IKA)\textsuperscript{228}. In addition to the hinge hydrogen bonds, the chlorine is directed towards the gatekeeper methionine (Met902 in the present structure) and the methoxy substituent extends toward the sidechain of Tyr904 in the hinge region. Interestingly, the “linker” segment that attaches the acrylamide adopts a distinctly different conformation as compared with the corresponding region of 8 in complex with EGFR\textsuperscript{T790M}. The phenyl group in the linker
pivots down and is in van der Waals contact with Leu956 in the floor of the ATP-binding cleft. This orientation is likely enabled by the longer aminomethylene moeity in 9, as compared with the ether linkage in compound 8. In addition, the acrylamide amide is positioned to hydrogen bond with the carbonyl of Arg953, also in the floor of the binding pocket.

Figure 7.4: Compound 9 (yellow stick) covalently binds to Cys909 at the GK+7 position of JAK3 (blue ribbons).

The syntheses of 9 was readily achieved with high-overall-yield in four steps starting from 2,4,5-trichloropyrimidine. The 4-chloride group was substituted with a 3-nitrobenzyl amine under basic conditions, followed by the substitution of the 2-chloride with a 2-methoxy-4-(4-methylpiperazin-1-yl)aniline under acidic conditions to give 11. The nitro group of 11 was reduced using hydrogenation and the resulted aniline was acylated to afford 9 (Scheme 7.1B). In order to elucidate the structural requirements to achieve potent and selective inhibition of JAK3 we prepared approximately 70 analogs. To approach this optimization in a systematic fashion, this chemotype was divided into three
moieties: tail (R₁), arm (R₂) and core (R₃), and each of these moieties were varied sequentially. The 2,4-disubstituted aniline tail moiety of 9 was replaced with different anilines, aliphatic amines or 4-aminopyrazoles; the 4-acrylamidobenzyl arm of 9 was substituted with functional groups at the double bond, phenyl ring or benzyl position; the pyrimidine core of 9 was modified at 5- or 6-position, or was replaced with a variety of bicyclic cores. All the analogs were profiled with the same JAK3 enzymatic assay and JAKs and ABL dependent Ba/F3 cell line assays. Additionally, the in vitro metabolic stability of some analogs was measured using mouse liver microsomes (MLM) to calculate half-lives (T₁/₂).

The first series of analogs focused on modification of the tail moiety (R₁) (Table 7.2). Substitutions at the 5-postion of the aniline (14, 15) resulted in a dramatic loss of potency. In contrast, removing the 2-methoxy group (16) enhanced potency over 5-fold against JAK3, however, it also increased the cytotoxicity toward other transformed Ba/F3 cell lines. Substitutions at the 3-position did not mitigate the cytotoxicity (17-19), nor did introduction of a fluorine at the 2-position (20). Compound 21 with a 4-morpholinylaniline tail potently inhibited JAK3 despite not having the 2-methoxy substitution. Furthermore, 21 showed comparable cellular selectivity and MLM T₁/₂ as 9 (T₁/₂ of 4.7 min.). However, 21 only showed moderate selectivity in biochemical assays, with 10~20-fold higher IC₅₀s against JAK1, JAK2 or TTK.
### Table 7.1: Enzymatic IC$_{50}$s of key compounds.

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Adding a 2- or 3-fluoro (22, 23) did not improve the kinase selectivity of 21 and also did not improve the MLM stability. 24 which contains two fluorine substituents at the 2- and 5-positions showed good selectivity but decreased potency against JAK3. 25, with a 3,5-difluorine, exhibited improved potency compared to 24 with good selectivity in cells, even though it showed moderate selectivity in enzymatic assays. Based on 25 several analogs (26-28) were made that exhibited increased potency, but none of them were as selective as 25. Aliphatic tails resulted in complete loss of potency (29-31). 32 with a 1-methyl-$1H$-pyrazol-3-amine tail showed increased potency, similar selectivity and MLM stability compared with 9; it also showed a 300-fold selectivity window over TTK. Addition of a 4-methyl group to the pyrazole ring (33) enhanced the MLM stability but decreased the potency over 10-fold. The isoxazol-4-amine tail in 34 is disfavored in terms of both potency and stability. Modifications were then focused on the 1-methyl group of the pyrazole tail. Replacement of the 1-methyl with a 1-difluoromethyl (35) or 1-difluoroethyl (36) maintained the potency and enhanced the MLM stability. However, bulky and hydrophobic substituents resulted in decreased MLM stability (37, 38). 39 with a 2-substituted $N$-methylacetamide exhibited noticeably improved MLM stability ($T_{1/2}$ of 14.3 min) and favorable selectivity albeit with only moderate potency against JAK3.
Nevertheless, other 2-substituted N-alkylacetamide groups (40-42) failed to improve the potency. Several analogs elaborated with a 2-substituted ethyl group (43-45) showed good potency but decreased MLM stability. To our surprise, 45 with a 2-methoxyethyl exhibited an IC$_{50}$ of 14 nM against JAK3 Ba/F3 cells, and over 500-fold selectivity over other Ba/F3 cell lines. 45 showed excellent selectivity with JAK3 IC$_{50}$ of less than 0.5 nM in fixed end point enzymatic assay, and over 70-fold higher IC$_{50}$ against JAK1 and over 100-fold higher IC$_{50}$s against JAK2, TYK2 or TTK (Table 7.1). 45 was profiled against a diverse panel of 468 kinases (DiscoveRX, KinomeScan$^1, 31$) at a concentration of 100 nM; the compound exhibited excellent overall kinase selectivity with selectivity scores, defined as the percentage of kinases with scores less than 5 (S(5))$^{31}$, of 0.01 (Figure 7.5). The results suggested 45 inhibits JAK3 most potently, and AURKA was likely to be the only off-target which we confirmed to be inhibited by 45 with IC$_{50}$ of 43 nM. 45 showed relatively poor MLM stability with $T_{1/2}$ of 3.3 min. Thus, several analogs with different 2-alkoxyethyl groups (46-48) were made to solve this problem. To our disappointment, all changes did not improve the MLM stability. Dimethyl substitution of the 2-methoxyethyl (49) is disfavored with respect to MLM stability as well as potency and selectivity. 50, with a hydroxyl group in the tail, showed 3-fold longer MLM $T_{1/2}$ compared with 45. Although 50 was not as selective as 45 in Ba/F3 cell lines, it was more potent than 9 with comparable selectivity in Ba/F3 cells. Further modification of 50 failed to improve the selectivity but led to decreased potency and MLM stability (51). In this series of analogs, 45 and 50 stood out with good selectivity or MLM stability, furthermore, we found 50 was actually one of the MLM metabolites of 45.
Figure 7.5: KinomeScan kinase selectivity profiles for Compound 45. Compound 45 were profiled at a concentration of 1 µM against a diverse panel of more than 468 kinases and mutants. Scores for primary screen hits are reported as a percent of the DMSO control (% control). The lower the Kd is likely to be, such that scores of zero represent strong hits. Scores are related to the probability of a hit but are not strictly an affinity measurement.

After optimization of the tail moiety, the SAR of the arm (R$_2$) was investigated (Table 7.3). Replacing the acrylamide with a propionamide (52) resulted in over an 80-fold decrease of potency against JAK3. Installation of a methyl group at α-position of the acrylamide (53) resulted in significant loss of potency and a shorter MLM $T_{1/2}$. Polar substitutions at the β-position of the acrylamide (54-56) also resulted in loss of potency. Addition of a fluorine ortho to the acrylamide in the phenyl ring (57) also decrease the potency dramatically. Switching the acrylamide from meta- to para- on the phenyl ring decreased the potency considerably in combination with the aniline tail of 9 (58), but did not affect the potency when used in conjunction with other tails (59-62). This change did however decrease the selectivity in some cases (59, 62). Analogs with a para-acrylamide generally showed comparable or diminished MLM stability. Finally, adding a methyl at the benzyl position (63, 64) reduced both potency and selectivity. In summary, the modifications of R$_2$ failed to further improve our JAK3 inhibitors. Several reported
inhibitors with similar acrylamide warheads (6, 7, 8 and 65\textsuperscript{238}) were also profiled and compared and none of them showed good inhibition against JAK3 Ba/F3 cells with IC\textsubscript{50}s over 1.0 µM.

Simultaneously we investigated the SAR of the core moiety (R\textsubscript{3}) (Table 1.4). We found that a thioether linkage at the 4-postion (66, 67) slightly increased the MLM stability but decreased the potency, and a tertiary amine linkage (-NMe-) decreased the potency even more dramatically (68). Next we investigated the effects of various substituents at the 5-position. We evaluated substituents that possessed electron withdrawing groups such as chlorine (69-72), electron donating groups (73-75) or simply a hydrogen atom (76). However, only 70 with a 5-bromo group showed good potency among these analogs. A 4,6-disubstituted pyrimidine core (77) also led to substantial loss of potency. Next we tried to cyclize the 4,5-substituents into a fused 5-membered or 6-membered ring (78-82), and found that only 78 with a pyrrolo[2,3-\textit{d}]pyrimidine core showed good potency against JAK3, but with a slightly decreased MLM stability. 81 with a pyrimido[4,5-\textit{d}]pyrimidinone core possessed a good IC\textsubscript{50} against JAK3 in the biochemical assay, but mediocre potency in the TEL-JAK3-transformed Ba/F3 cells. In the end, modifications at the 6-position of the pyrimidine core (83, 84) were fairly disfavored. These results suggested that 5-chlorine is the most suitable, and the secondary amine (-NH-) is a satisfactory linkage at 4-postion; some bicyclic cores did improve the inhibitor modestly, and modifications at 6-position are not tolerated.

Based on the SAR results above, we selected 9, 45 and 50 as our optimal JAK3 inhibitors, and investigated their inhibitory activity on JAK3-dependent signaling. In the TEL-JAK3 Ba/F3 cells, STAT5 is a direct substrate of JAK3 and is constitutively
phosphorylated. Compound 2 at 300 nM almost fully inhibited the phosphorylation of STAT5 (p-STAT5), while 9 at 300 nM completely abolished p-STAT5, and so did 45 or 50 at 100 nM. (Figure 7.6). These results are consistent with the IC_{50}s in JAK-transformed Ba/F3 proliferation assays.

Figure 7.6: Compound 9, 45, and 50 are covalent, irreversible JAK3 inhibitors. TEL-JAK3 Ba/F3 cells were treated with 2, 9 (1 µM), 45 or 50 (300 nM) for 3 h, washed extensively with PBS, allowed to recover for 4 h, then lysed and subjected to western blot for phospho-STAT5 and total STAT5 (A). TEL-JAK3 Ba/F3 cells were treated 2, 9 (1 µM), 45 or 50 (300 nM) for 3 h, and the resulting cell lysates were treated with 85 (B) (1.0 µM, 1 h), followed by pull-down with streptavidin beads and immunoblotting with anti-JAK3 antibody (C).

In order to confirm that 45 and 50 are bonafide covalent inhibitors in analogy to compound 9 we performed cellular ‘wash-out’ experiments. JAK3 Ba/F3 cells were treated with these four inhibitors at various concentrations for 3 h, the cells were washed extensively with PBS and then allowed to recover for 4 h. Western blot of the cellular lysates revealed that 9, 45 or 50 were capable of sustained inhibition of p-STAT5 after the washout, whereas the reversible inhibitor 2 was not (Figure 7.7A). To monitor the degree of JAK3 ‘target engagement’, a biotinylated version of 45 (85) was designed with a biotin tethered via a flexible PEG linker at the tip of 45’s tail moiety (Figure 7.7B). We confirmed that 85 maintained similar biochemical potency and selectivity for JAK3 (Table
85 showed selective but weak potency against JAK3 Ba/F3 cells with an IC$_{50}$ of 1.3 µM presumably due to poor membrane permeability. We demonstrated that streptavidin mediated pulldown of 85 in cell lysates allowed for efficient recovery of JAK3 as assessed by western blotting. Consistent with the wash-out results, 85 strongly labeled JAK3 when cells were treated with the reversible inhibitor 2 but not when cells were treated with acrylamide modified inhibitors 9, 45 or 50 (Figure 7.7C). Compound 9, 45 or 50 were further demonstrated to be covalent inhibitors using electrospray mass spectrometry, where incubation of recombinant JAK3 kinase domain with inhibitor resulted in addition of the expected molecular weight (Figure 7.8). Subsequent protease digestion and LC/MS$^2$ analysis identified the only peptide (residues LVMEYLPSC^LR) modified by 9, 45 or 50 to contain Cys909 which matches what is illustrated in the JAK3/9 co-crystal structure. Cumulatively these results provide strong evidence that 9, 45 and 50 are all irreversible, covalent inhibitors and that Cys909 of JAK3 is the only labeled site.
Figure 7.7: Compound 9, 45, and 50 react quantitatively with JAK3. Raw (A, C, E, G) and deconvoluted (B, D, F, H) mass-spectra obtained for JAK3 after treatment with DMSO (A, B), 9 (C, D), 45 (E, F), or 50 (G, H).
Figure 7.8: Compound 9, 45 and 50 potently and selectively inhibited JAK3-dependent signaling in BMDMs cells. BMDMs cells were pre-incubated with the indicated concentrations of 9 (A), 45 (B) or 50 (C) for 3 h. Cells were then stimulated with the indicated cytokines (IL-4 at 10 ng/mL, INFβ at 500 U/mL, GM-CSF at 10 ng/mL, IL-10 at 100 ng/mL) for 30 min then lysed. The levels of tyrosine phosphorylation on the appropriate STAT protein were determined by immunoblotting. Levels of total ERK1/2 were also examined to show equal loading. The signaling cascades were described in D.
Compounds 9, 45 and 50 were further evaluated for their ability to inhibit JAK3 kinase activity in a variety of other cell types. We evaluated the ability of 9, 45 or 50 to inhibit signaling following stimulation of primary mice bone marrow derived macrophage (BMDMs) with a panel of 4 different cytokines based on their requirement for different cytokines (Figure 7.8). Among these cytokines, only signaling via IL-4, whose receptor binds JAK1 and JAK3,\(^{239}\) is dependent on JAK3. In contrast, GM-CSF, which signals via JAK2, and IL-10 and IFNβ, which signal via JAK1 and TYK2, are JAK3 independent.\(^{240}\) 9, 45 or 50 completely inhibited IL-4 induced p-STAT6 at a concentration of 500 nM and only partially inhibited INFβ-induced p-STAT1 at a concentration of 5.0 µM. None of our inhibitors detectably reduced the p-STAT5 level stimulated by GM-CSF or p-STAT3 induced by IL-10 at a concentration 5.0 µM. In a similar way, 9, 45 and 50 exhibited consistent potency and selectivity for JAK3 in leukemia cancer cell lines: T-ALL1 and OCL-AML5 (Figure 7.9). In T-ALL1 cells, IL-2 induced STAT5 phosphorylation was completely inhibited by 9 at a concentration 1.0 µM or by 45 or 50 at a concentration of 100 nM (Figure 7.9A). However, in OCL-AML5 cells the GM-CSF induced STAT5 phosphorylation was maintained in the presence of the inhibitors up to a concentration of 10 µM (Figure 7.9B). In contrast, previously reported pan-JAK inhibitors 1 and 2 could abolish p-STAT5 in both stimulated cell lines at 1.0 µM. Based on these results compounds 9, 45 and 50 are capable of efficiently inhibiting JAK3 and specifically blocking the JAK3-dependent signaling pathway in human cells at sub-micromolar concentrations.
Figure 7.9: Compound 9, 45 and 50 potently and selectively inhibited JAK3-dependent p-STAT5 in TALL-1 cells. Whereas pan-JAK inhibitors 1 and 2 also potently inhibited JAK2-dependent p-STAT5 in OCL-AML5 cells. TALL-1 (A) or OCL-AML5 (B) cells were pre-incubated with the indicated concentrations of indicated inhibitors for 3 h. Cells were then stimulated with 10 ng/mL of IL-2 (A) or GM-CSF (B) for 30 min then lysed. The p-STAT5 levels were determined by immunoblotting. Levels of total STAT5 were also examined to show equal loading.

We next evaluated the pharmacokinetic properties of 9 in mice following intravenous and oral delivery. Compound 9 demonstrated reasonable pharmacokinetic properties, with moderate $T_{1/2}$ of 1.4 h, AUC value of 795 ng*hr/mL following a 10 mg/Kg oral dose and good oral bioavailability of 66% (Table 7.5). This suggests that 9 may be a suitable probe for future murine efficacy studies.

Table 7.2: SAR of $R_1$. 

![Table 7.2: SAR of $R_1$.](image)
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Table 7.3: SAR of R₂.

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Table 7.4: SAR of R₃.

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Table 7.5: Pharmacokinetic properties of 9.

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<th>C(_{\text{max}}) (ng/mL)</th>
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<th>CL (mL/min/kg)</th>
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7.2.4. Discussion

Building on our covalent T790M EGFR inhibitor 8 we designed and synthesized new analogs aiming to target the analogous cysteine residue in JAK3. Among those analogs compound 9 was identified as a potent and selective JAK3 inhibitor compared with current reversible JAK inhibitors. We determined a 2.9 Å co-crystal structure of JAK3 with 9, which revealed that 9 covalently binds to Cys909 of JAK3 as expected. With 9 as a lead, we designed and synthesized dozens of analogs to intensively study the SAR with regard to each moiety of this scaffold. These analogs were tested in enzymatic assays for their potency against JAK3 and profiled against a panel of transformed-Ba/F3-cell for their potency and selectivity in a cellular context. Based on these studies we discovered that a 2-methoxy group in the aniline tail of 9 is disfavored by JAK3 presumably due to a disfavorable interaction with the bulky side chain of Tyr904 located at the GK+2 position. Hydrophobic or less polar tails are better for selectivity for JAK3 presumably because they have weaker non-covalent affinity for the kinase thereby allowing covalent bond formation to be a more important contributor to the potency. The acrylamidobenzyl arm moiety of 9 is significant for the potency against JAK3 and sensitive to changes, its specific length and flexibility are critical to enable the inhibitors to efficiently form a covalent bond with Cys909 of JAK3. The H-bond between the acrylamide of 9 and the carbonyl of Arg953 fixed the conformation of the acrylamide and left no space to accommodate substituents. The chlorine atom in the pyrimidine core is responsible for
both potency and selectivity with its interaction with Met902 of JAK3. Modifications at the 6-position of the pyrimidine core were fairly disfavored as they collided with the carbonyl of Glu903 3.3Å away. Some of these analogs were also evaluated for their MLM stability, the results indicated that more lipophilic analogs with aliphatic non-polar substituents tend to have a shorter half-life, maybe resulting from their enhanced binding with phospholipid in microsomes.241

Among these analogs 9, 45 and 50 stood out with overall favorable potency, selectivity and MLM stability. They all exhibited efficient and selective inhibition of JAK-dependent signaling within different contexts. So far 45 is our most selective JAK3 inhibitor with at least 70-fold selectivity over other JAKs in biochemically assays, and at least 500-fold selectivity in JAK-transformed Ba/F3 cells. 50 has improved MLM stability and comparable selectivity to 9 in cellular assays. In addition to the JAK3/9 co-crystal structure, all the three inhibitors were demonstrated to covalently modify Cys909 of JAK3 based upon mass spectrometry, wash-out and pull-down experiments We developed a biotinylated probe 85 which was demonstrated to be a useful reagent for establishing “target-engagement” in cellular assays.

In conclusion, the covalent JAK3 inhibitors 9, 45, 50 and the biotinylated probe 85, provide a set of useful tools to pharmacologically interrogate JAK3-dependent biology. Compound 45 is a highly potent and selective biochemical and cellular inhibitor of JAK3 and is an ideal reagent for cell biological studies. Compound 9 combines favorable target profile with good pharmacokinetic properties which should enable its use in future animal studies. Finally, the structure-activity relationships with respect to JAK3 combined with
the co-crystal structure will serve as an excellent foundation for future JAK3 inhibitor development efforts.

7.3. Materials and Methods

Chemistry. Unless otherwise noted, reagents and solvents were obtained from commercial suppliers and were used without further purification. $^1$H NMR spectra were recorded on 600 MHz (Varian AS600), and chemical shifts are reported in parts per million (ppm, $\delta$) downfield from tetramethylsilane (TMS). Coupling constants ($J$) are reported in Hz. Spin multiplicities are described as s (singlet), br (broad singlet), d (doublet), t (triplet), q (quartet), and m (multiplet). Mass spectra were obtained on a Waters Micromass ZQ instrument. Preparative HPLC was performed on a Waters Sunfire C18 column (19 x 50 mm, 5µM) using a gradient of 15-95% methanol in water containing 0.05% trifluoroacetic acid (TFA) over 22 min (28 min run time) at a flow rate of 20 mL/min. Purities of assayed compounds were in all cases greater than 95%, as determined by reverse-phase HPLC analysis.

2,5-Dichloro-N-(3-nitrobenzyl)pyrimidin-4-amine (10). 2,4,5-Trichloropyrimidine (112 µL, 1.0 mmol), (3-nitrophenyl)methanamine hydrochloride salt (227 mg, 1.2 mmol), and $N,N$-diisopropylethylamine (DIEA, 530 µL, 3.0 mmol) were combined in dioxane (5 mL) and stirred overnight. The mixture was then diluted with ethyl acetate and washed with water and brine, dried over Na$_2$SO$_4$, filtered and concentrated. The crude product was purified by column chromatography (hexane:ethyl acetate = 1:1) to yield 270 mg (80%) of 10 as a white solid. $^1$H NMR (600 MHz, CDCl$_3$) $\delta$ 8.21 (s, 1H), 8.18 (d, $J = 8.4$ Hz, 1H), 8.10 (s, 1H), 7.71 (d, $J = 7.8$ Hz, 1H), 7.56 (dd, $J = 7.8$, 7.8 Hz, 1H), 5.94 (br, 1H), 4.84 (d, $J = 6.0$ Hz, 1H). MS (ESI) m/z 299 (M+H)$^+$. 
5-Chloro-N²-(2-methoxy-4-(4-methylpiperazin-1-yl)phenyl)-N⁴-(3-nitrobenzyl)pyrimidine-2,4-diamine (11). To 10 (150 mg, 0.5 mmol) and 2-methoxy-4-(4-methylpiperazin-1-yl)aniline (166 mg, 0.75 mmol) in sec-butanol (5 mL) was added trifluoroacetic acid (57 µL, 0.75 mmol) and the mixture was stirred overnight at 100 °C. The mixture was then concentrated, neutralized with ammonia in methanol and purified by column chromatography (dichloromethane:methanol = 10:1) to yield 184 mg (76%) of 11 as a pale-yellow solid. ¹H NMR (600 MHz, CD₃OD) δ 8.23 (s, 1H), 8.09 (d, J = 8.4 Hz, 1H), 7.84 (s, 1H), 7.70 (d, J = 7.8 Hz, 1H), 7.65 (d, J = 9.0 Hz, 1H), 7.54 (dd, J = 7.8, 7.8 Hz, 1H), 6.60 (d, J = 3.0 Hz, 1H), 6.36 (dd, J = 9.0, 3.0 Hz, 1H), 4.75 (s, 1H), 3.83 (s, 3H), 3.15 (m, 4H), 2.64 (m, 4H), 2.36 (s, 3H). MS (ESI) m/z 484 (M+H)+.

N-(3-(((5-chloro-2-((2-methoxy-4-(4-methylpiperazin-1-yl)phenyl)amino)pyrimidin-4-yl)amino)methyl)phenyl)acrylamide (9). To 11 (97 mg, 0.2 mmol) in MeOH (20 mL) was added 1 mL Raney nickel suspension in MeOH. The reaction mixture was stirred for 3 h under 1 atm of hydrogen. The mixture was then filtered with Celite, and the filtrate was concentrated and dried under vacuum to give a crude product as a white solid. To the obtained white solid in DMF (2 mL) was added DIEA (53 µL, 0.3 mmol), the stirred mixture was then cooled to -60 °C, and acryloyl chloride (17.8 µL, 0.22 mmol) was added dropwise. The reaction mixture was stirred at -60 °C for 10 min, allowed to recover to RT (room temperature) gradually in 30 min, and purified by reverse phase HPLC to give 88 mg (TFA salt, 71% for 2 steps) of 9 as a white solid. ¹H NMR (600 MHz, DMSO-d₆) δ 10.07 (s, 1H), 7.82 (s, 1H), 7.73 (m, 1H), 7.59 (s, 1H), 7.52 (d, J = 8.4 Hz, 1H), 7.47 (d, J = 7.8 Hz, 1H), 7.29 (s, 1H), 7.18 (dd, J = 7.8, 7.8 Hz, 1H), 6.90 (d, J = 7.2 Hz, 1H), 6.48 (s, 1H), 6.44 (dd, J = 16.8, 10.2 Hz, 1H), 6.31 (d, J = 8.4 Hz, 1H), 6.25 (d, J = 16.8 Hz,
1H), 5.75 (d, J = 10.2 Hz, 1H), 4.56 (d, J = 6.0 Hz, 2H), 3.78 (s, 3H), 3.04 (m, 4H), 3.01 (m, 4H), 2.22 (s, 3H). MS (ESI) m/z 508 (M+H)⁺.

Compound 14-64, 66-85 were synthesized with same procedures as 9, 3 and 8 were synthesized as reported.²²⁶, ²³⁴ Compound 1, 2, 6, 7, 12, 13 and 65 were from Selleckchem, 4 was from Santa Cruz, and 5 was from Millipore.

Protein expression and purification. Human JAK3 kinase domain (residues 811-1124) was expressed and purified as described previously³² except that JAK3 was co-expressed with human c-SRC (residues 86-536) to achieve more consistent phosphorylation of JAK3.

Crystallization and structure determination. Crystals of JAK3 in complex with 9 were prepared using the hanging drop vapor diffusion method; JAK3 at a concentration of 5 mg/mL was incubated for 30 min with 500 μM compound 9, then added to an equal volume of well solution (0.1 M Bis-tris pH 6.5, 0.2 M ammonium sulfate, 16% PEG 3350, 5 mM TCEP) and equilibrated over well solution at 4 °C. Crystals were looped into cryoprotectant solution (0.1 M Bis-tris pH 6.5, 0.2 M ammonium sulfate, 20 % PEG 3350, 15% glycerol, 5 mM TCEP) for 30-60 sec, then flash-frozen in liquid nitrogen. X-ray diffraction data were collected at APS beamline 24-ID-E (NE-CAT), processed with XDS,²⁴² and scaled with Scala.²⁴³, ²⁴⁴ The structure was solved by molecular replacement with Phaser²⁴⁴ using the JAK3 kinase domain as a search model (PDB ID 1YVJ).³² Manual refitting of the crystallographic model was performed with Coot²⁴⁵ and refinement was performed with Phenix²⁴⁶ and BUSTER (version 2.10.2. Cambridge, United Kingdom: Global Phasing Ltd).²⁴⁷ Topology and parameter files for compound 9 were generated with PRODRG.²⁴⁸
**Mass spectrometry.** JAK3 protein (5 µg) was labeled with a 10-fold excess of inhibitor or DMSO for 30 minutes at 4 ºC. After labeling, proteins were desalted using 0.5 mL Zeba spin desalting columns (Thermo Fisher Scientific). Analysis of intact protein was performed essentially as described. Briefly, ~5 µg protein was injected onto a self-packed reversed-phase column (500 µm inner diameter, 5 cm of POROS 50R2 resin). After washing to remove salts, protein was eluted with an HPLC gradient (0%–100% B in 1 min, A = 0.2 M acetic acid in water, B = 0.2 M acetic acid in acetonitrile, flow rate = 10 µL/min) into a linear ion trap mass spectrometer (LTQ, Thermo Fisher Scientific, San Jose, CA). Data were acquired in profile mode scanning m/z 300-2000. Mass spectra were deconvoluted using MagTran software (version 1.03b2). Sites of covalent modification were identified using a “bottom-up” strategy. Desalted proteins were reduced with tris(2-carboxyethyl)phosphine (10 mM, 10 min room temperature), alkylated with iodoacetamide (20 mM, 30 min room temperature in dark), and digested with trypsin for 4 h at 37 ºC. Digests were analyzed by nanoLC-ESI-MS as described with modifications. Peptides were loaded onto a self-packed pre-column (4 cm POROS10R2), resolved on an analytical column (30 µm I.D., packed with 12 cm C18) and eluted into the mass spectrometer (LTQ Orbitrap XL, Thermo Fisher Scientific) using an HPLC gradient (Waters NanoAcquity, Milford, MA; 0%–35% B in 60 min, A = 0.2 M acetic acid in water, B = 0.2 M acetic acid in acetonitrile, flow rate = ~30 nL/min). The instrument was operated in data dependent mode such that the top 10 most abundant precursors were subjected to MS/MS (electron multiplier detection, relative collision energy 35%, q = 0.25). Raw data files were converted to .mgf using in-house software and searched using Mascot 2.2.1 against a forward-reverse human refseq database.
Search parameters specified variable oxidation of methionine, variable inhibitor modification (9, 45, or 50) of cysteine, and fixed carbamidomethylation of cysteine (i.e. cysteines are considered carbamidomethylated or inhibitor labeled). To confirm labeling sites, internally calibrated HCD spectra (image current detection, resolution at \( m/z \) 200 = 15,000, relative collision energy 35%) were acquired using an Orbitrap Fusion mass spectrometer (Thermo Fisher Scientific). For these experiments, LC parameters were similar to those described above, except that the analytical column was packed with 50 cm of C18 and peptides were eluted with a gradient of 0-35% B in 90 min.

**Mouse liver microsomal stability.** The MLM assays were previously reported and are commercially available from Scripps Florida.\(^{237}\)

**Ba/F3 cell viability assays.** Ba/F3 derivatives expressing various oncogenic fusion kinases, TEL-JAK1, TEL-JAK2, TEL-JAK3 and TEL-ABL were described previously.\(^{231}\) Ba/F3 cells transformed by TYK2\(^{5957D}\) were generated as previously described.\(^{233}\) These cells were maintained in RPMI-1640 medium (GIBCO) supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich) and penicillin/streptomycin (Invitrogen). For cell viability assays, the cells were plated at a density of 10,000 cells per well in a 96-well white plate and incubated with DMSO or increasing concentrations of drugs. At 72 h after the initiation of treatment, the relative cell viability was determined using the Cell Titer Glo assay (Promega) and reported as a percentage of the DMSO control. Concentration values for 50% inhibition (IC\(_{50}\)) of cell viability were determined with GraphPad Prism software.

**Immunoblotting analysis and washout experiment.** Whole-cell lysates were prepared in RIPA buffer (Cell Signaling) with FOCUS™, ProteaseArrest™ (G-Biosciences) and
Phosphatase Inhibitor Cocktail Set II (EMD Millipore). Immunoblotting was performed with each of specific antibodies to STAT5, phospho-STAT5 (Tyr694), α-tubulin (Cell Signaling) and JAK3 (Santa Cruz, #sc-513). For washout experiment, cells were incubated with JAK3 inhibitors or DMSO for 3 h, washed with PBS three times, maintained in RPMI-1640 medium with 10% of FBS for 4 h, and then the protein was harvested.

**Streptavidin pulldown experiment.** Ba/F3 cells transformed by TEL-JAK3 were incubated with JAK3 inhibitors or DMSO for 3 h, and then lysed with Pierce IP lysis buffer (Thermo Scientific) with Halt™ protease inhibitor cocktail (Thermo Scientific). The lysates were treated with 85 (5 µM) at 4 °C overnight, and then further incubated at room temperature for 3 h. The solution was mixed with Streptavidin beads (25 µL, Sigma) and incubated with rotation at 4 °C for 2 h. The beads were washed five times using the lysis buffer with 4 M urea (1 mL), and then boiled in the presence of Lammlli sample buffer with 2-mercaptoethanol. The eluted proteins were immunoblotted with each of specific antibodies.

**Cell stimulations and Western Blots. (Figure 7.8, 7.9).**

Primary bone marrow derived macrophages (BMDMs) were prepared as described.253 Cells were maintained on bacterial grade DMEM supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 U/ml of penicillin G, 100 µg/ml of streptomycin, 0.25 µg/ml of amphotericin (Invitrogen), and 5 ng/ml of macrophage colony-stimulating factor (M-CSF; PreProTech) for 7 days. Cells were then replated onto tissue culture grade plastic and used the following day. Following stimulation cells were lysed into SDS sample buffer and run on 10% polyacrylamide gels according to standard techniques. Proteins were transferred onto nitrocellulose membranes and blotted with antibodies against total
ERK1/2, p-Y641 STAT6, p-Y701 STAT1, pY701 STAT3 or pY694 STAT5 (all from Cell Signaling Technology). The experiments in cancer cell lines were done with similar procedures.

**In Vivo Pharmacokinetic Studies.** Male Swiss albino mice were dosed via tail vein (intravenous, 0.1% v/v Tween 80, 0.5% w/v NaCMC in water at a dose of 10 mg/kg) or via oral gavage (suspensions in 5% NMP, 5% solutol HS in normal saline intravenously via tail vein at a dose of 2 mg/kg). Blood samples were collected at 0.08, 0.25, 0.5, 1, 2, 4, 8 and 24 h (i.v.) and at Predose, 0.25, 0.5, 1, 2, 4, 6, 8 and 24 h (p.o.). Plasma samples were separated by centrifugation of whole blood and stored below -70ºC until bioanalysis. All samples were processed for analysis by protein precipitation using acetonitrile and analyzed with fit-for-purpose LC/MS/MS method (LLOQ, 1.06 ng/mL). Pharmacokinetic parameters were calculated using the non-compartmental analysis tool of WinNonlin Enterprise software (version 6.3).

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**Abbreviations.** TYK2, tyrosine kinase 2; IL-2, interleukin-2; NK cells, natural killer cells; T-ALL, T-cell acute lymphoblastic leukemia; T-PLL, T-cell prolymphocytic leukemia; EGFR, epidermal growth factor receptor; BTK, Bruton’s tyrosine kinase; MAP2K7, mitogen-activated protein kinase kinase 7; FLT3, fms-related tyrosine kinase 3; TEC, tyrosine protein kinase; ABL, Abelson murine leukemia viral oncogene homolog; TTK,
TTK protein kinase; AURK, aurora kinase; INFβ, interferon beta; GM-CSF, Granulocyte-macrophage colony-stimulating factor; AUC, area under the curve.
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